Vitamin B6 Status-Dependent Immunomodulation by Caramel Colour III

Geert Houben

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Vitamine B6 Status-Afhankelijke Immunomodulatie door Caramel Kleurstof III

(met een samenvatting in het Nederlands)

Proefschrift

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Gerardus Franciscus Houben

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Promotor: Prof. Dr. W. Seinen

Co-promotor: Dr. A.H. Penninks

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Contents

Chapter 1

General Introduction

Caramel Colour

Caramel Colour (E150) refers to a large number of complex products, produced by the controlled heating of carbohydrates together with defined ranges of specified reactants under varying conditions of temperature and pressure. These dark-brown to black viscous liquids with the characteristic odour of burnt sugar and a somewhat bitter taste are among the oldest colourants known to be added to human food. Their use accounts for the majority (about 95% by weight) of the permitted colour additives used in food. Nevertheless, because of their complex composition, Caramel Colours have remained chemically rather ill-defined. The chemical composition of Caramel Colours depends upon the process conditions and the reactants used in their manufacture. Based on the reactants used for their production, commercial Caramel Colours have been classified into four classes which differ in their chemical and functional properties as well as their applications (Ward, 1976; FAO, 1977; Noltes and Chappel, 1985; JECFA, 1986): Caramel Colour I, also known as Plain Caramel Colour or Spirit Caramel, is produced in the presence of small amounts of acids, alkalis, or other technical agents, but not including ammonia or sulphite compounds. Caramel Colour II, also referred to as Caustic Sulphite Process Caramel or Ice Cream Caramel, is produced in the presence of sulphite compounds, however excluding ammonia compounds. Caramel Colour III, synonyms of which are Ammonia Process Caramel, Ammonia Caramel, Beer Caramel and Bakers Caramel, is produced in the presence of small amounts of specified ammonia compounds with or without other technical agents but not including sulphite compounds. Finally, Caramel Colour IV, also called Sulphite Ammonia Process Caramel, Soft Drink Caramel or Acid Proof Caramel, is produced in the presence of ammonia compounds and sulphite compounds, with or without other technical agents.

As indicated, considerable differences in chemical composition can be noted between Caramels belonging to different classes. However, within each class, a variation in the chemical composition of Caramel Colours can also be noted (Patey *et al.*, 1985a; Patey *et al.*, 1985b). During the preparation of Caramel Colours, the bulk of constituents which are formed are low-molecular-weight compounds, whereas polymeric products constitute a lesser amount. In the case of Caramel Colour III, over one hundred separate low-molecular-weight compounds were reported (Patey *et al.*, 1985b). Among these compounds are various nitrogen-containing heterocycles such as imidazoles, pyrazines and pyridines (Wilks *et al.*, 1977; Kröplien *et al.*, 1985; Patey *et al.*, 1985b; Patey *et al.*, 1987). Ammonia Caramel Colours constitute a class of

Caramel Colour commonly used in many products, including various bakery products, soya-bean sauces, brown sauces, gravies, soup aromas, brown (dehydrated) soups, brown malt caramel blend for various applications, vinegars and beers, particularly in certain dark-brown beers. Their use accounts for 20 to 25% of the total use of Caramel Colours in the USA and for about 50% in Europe.

Toxicity studies on Caramel Colours

Numerous studies have been conducted on the safety of Caramel Colours. Studies on Class I, II and IV Caramel Colours indicated that these Caramel Colours produce no toxicologically significant effects in mammals (Noltes and Chappel, 1985). However, it has been demonstrated that administration of Caramel Colour III can cause a reduction in total white blood cell counts in rats (Evans *et al.*, 1977; Gaunt *et al.*, 1977; Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988), due to reduced lymphocyte counts. The effect of Caramel Colour III in rats was found to occur only when the animals were fed a diet low in vitamin B6. Addition of vitamin B6 to the diet of Caramel Colour III-exposed rats prevented the reduction in the number of lymphocytes (Noltes and Chappel, 1985; Conway and Paine, 1988; Sinkeldam *et al.*, 1988; Gobin and Paine, 1989). Following the isolation and identification of 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI: $C_9H_{14}N_2O_5$, M=230.22; Figure 1.1)

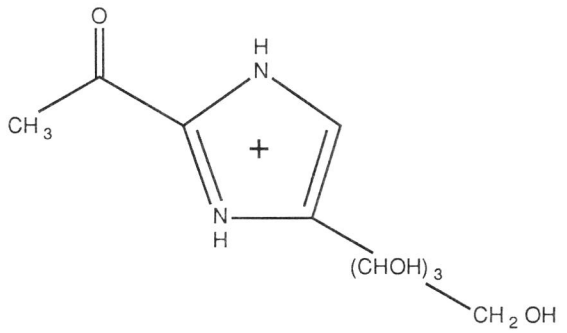


Figure 1.1: Structure of 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI: $C_9H_{14}N_2O_5$, M=230.22)

from Caramel Colour III (Kröplien *et al.*, 1985), comparative studies indicated that this was the major, if not the only component of Caramel Colour III responsible for the reduction in the number of blood lymphocytes (Noltes and Chappel, 1985; Conway and Paine, 1986c; Conway and Paine, 1988; Sinkeldam *et al.*, 1988).

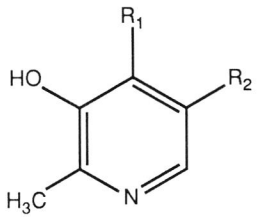
In the past 10 years, several studies were conducted in an attempt to elucidate the mechanism responsible for the lymphocyte reduction. Most of these studies concentrated on the role of vitamin B6 (Spector and Huntoon, 1982; Schreurs and Sinkeldam, 1985; Conway and Paine, 1986a; Conway and Paine, 1986b; Conway and Paine, 1988; Sinkeldam *et al.*, 1988; Gobin and Paine, 1989), but so far, not much progress was made in this respect.

Vitamin B6

Vitamin B6 refers to all derivatives of 3'-hydroxy-2'-methyl-pyridine which have the same qualitative biological activity in the rat as pyridoxine (IUPAC-IUB Commission, 1973). There are 6 naturally occurring forms of vitamin B6: pyridoxal (PL), pyridoxamine (PM), pyridoxine (PN), and their 5'-phosphate esters pyridoxal 5'-phosphate (PLP), pyridoxamine 5'-phosphate (PMP) and pyridoxine 5'-phosphate (PNP) (Figure 1.2). The derivative 4'-pyridoxic acid (PA; Figure 1.2) is the main degradation product of vitamin B6 and has no vitamin activity. The main coenzyme active form of vitamin B6 is PLP. This coenzyme is required for many of the enzyme-catalysed reactions of amino acids, including decarboxylations, transaminations, racemizations, β- and γ-eliminations and several others (Friedrich, 1988). The PLP-catalysed reactions with amino acids start with Schiff's base formation between PLP and amino groups of the amino acids (Friedrich, 1988). To a lesser extent, PMP is active as coenzyme. Animals are not able to synthesize vitamin B6 *de novo*, but they can interconvert the naturally occurring forms of the vitamin (Figure 1.3).

Phosphorylated forms of vitamin B6 do not easily pass cell membranes (Snell and Haskell, 1971; Lumeng *et al.*, 1980). After oral intake, 5'-phosphate esters of vitamin B6 are hydrolysed in the lumen of the intestinal tract by membrane associated alkaline phosphatase activity (Middleton, 1982), and the non-phosphorylated forms are rapidly absorbed by passive diffusion (Buss *et al.*, 1980; Hamm *et al.*, 1979; Mehansho *et al*, 1979; Middleton, 1982). Kinase mediated metabolic trapping and binding to proteins are indicated to play a role in the absorption of vitamin B6 (Rose, 1981; Friedrich, 1988; Bowman *et al.*, 1989).

In addition, metabolic conversion of PN into PL and PLP has been reported to take place in the intestinal tissue (Sakurai *et al.*, 1988).



R_1	$R_2 = CH_2OH$	$R_2 = CH_2OPO(OH)_2$
CH_2OH	pyridoxine (PN)	pyridoxine 5'-phosphate (PNP)
H-C=O	pyridoxal (PL)	pyridoxal 5'-phosphate (PLP)
H_2-C-NH_2	pyridoxamine (PM)	pyridoxamine 5'-phosphate (PMP)
COOH	4'-pyridoxic acid (PA)	

Figure 1.2: Structure of naturally occurring forms of vitamin B6 and 4'-pyridoxic acid

In mammals, PLP is bound to various proteins of the tissues and body fluids, which protects vitamin B6 from degradation and serves to store the vitamin (Friedrich, 1988). The major transport forms of vitamin B6 in blood plasma are PL and PLP, the latter almost completely bound to plasma proteins (Anderson *et al.*, 1974). Non-phosphorylated forms of vitamin B6 are also taken up by erythrocytes in large quantities by passive diffusion, the concentration gradient being maintained mainly by binding of the vitamin to hemoglobin (Friedrich, 1988). Kinase mediated metabolic trapping is indicated to be involved in cellular uptake and accumulation of vitamin B6 in various tissues and types of cells (Ink and Henderson, 1984; Friedrich, 1988; Bowman *et al.*, 1989). After uptake of non-phosphorylated forms of vitamin B6 into cells, conversion into the common coenzyme form PLP takes place and PLP may bind to the apoenzyme of PLP-dependent enzymes (see also Chapter 5 of this thesis). For more information on biochemical functions or enzymes in which vitamin B6 is

involved as a coenzyme, it is referred to Friedrich (1988). Because phosphorylated forms of vitamin B6 do not easily pass cell membranes and transport of vitamin B6 is influenced by kinase mediated metabolic trapping, the role of pyridoxal kinase, the enzyme catalysing the phosphorylation of PL, PM and PN, is significant in the synthesis of the coenzymatic active form PLP, as well as in primary uptake and transport between tissue cells and cell compartments (Friedrich, 1988).

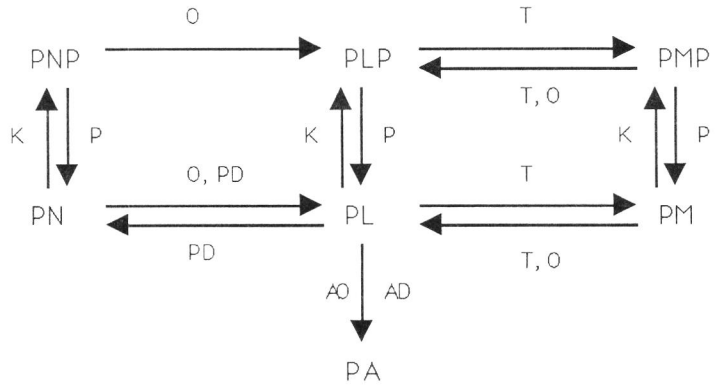


Figure 1.3: Interconversions and degradation of B6 vitamirs

Enzymes: K: pyridoxal kinase; P: phosphatase; O: pyridoxamine phosphate oxidase or pyridoxine oxidase; T: aminotransferase; PD: pyridoxine dehydrogenase (not in mammals); AO: aldehyde oxidase; AD: aldehyde dehydrogenase. *Compounds:* PL: pyridoxal; PM pyridoxamine; PN: pyridoxine; PLP: pyridoxal 5'-phosphate; PMP: pyridoxamine 5'-phosphate; PNP: pyridoxine 5'-phosphate; PA: 4'-pyridoxic acid.

Sources: Wilson & Davis, 1983; Driskell, 1984; Ink & Henderson, 1984, and Friedrich, 1988.

Caramel Colour III Immunotoxicity and Vitamin B6

There is evidence indicating that Caramel Colour III may affect mammalian vitamin B6 metabolism. It was demonstrated that Caramel Colour III may contain a competitive inhibitor of pyridoxal kinase (Spector and Huntoon, 1982; Schreurs and Sinkeldam, 1985). From the information presented in the previous paragraph, it is obvious that inhibition of pyridoxal kinase may interfere with the synthesis of the major coenzymatic active form of vitamin B6, PLP, as well as with transport of vitamin B6. Decreased plasma PLP levels in Caramel Colour III-exposed rats were reported indeed (Schreurs and Sinkeldam, 1985; Sinkeldam *et al.*, 1988). However, exposure to THI was not found to be associated with obvious changes in the vitamin B6 status (Sinkeldam *et al.*, 1988), while Caramel Colour III-induced lymphopenia was not always accompanied by changes in vitamin B6 status either (Conway and Paine, 1986a). Moreover, the pyridoxal kinase inhibiting compound in Caramel Colour III (Spector and Huntoon, 1982) was indicated to have a molecular weight different from that of THI (Kröplien *et al.*, 1985): about 360 vs 230, respectively.

Although the relevance of the effects of Caramel Colour III on vitamin B6 metabolism with respect to the lymphopenia observed remained unclear, studies in rats with the vitamin B6 antagonist 4'-deoxypyridoxine (DOP) demonstrated similar effects on lymphocyte counts and on plasma PLP levels (Schreurs and Sinkeldam, 1985; Conway and Paine, 1986a; Sinkeldam *et al.*, 1988). As in the case of treatment with Caramel Colour III, the decrease in the number of lymphocytes was apparent within 3 days (Schreurs and Sinkeldam, 1985; Sinkeldam *et al.*, 1988). Lymphocyte counts may also be decreased simply by feeding a vitamin B6 deficient diet with a pyridoxine content of 1 ppm or below. However, this effect appeared to be maximal only after 3 months (Schreurs and Sinkeldam, 1985). Classical signs of a vitamin B6 deficiency have not been observed in toxicity studies with Caramel Colour III or THI.

The influence of vitamin B6 on the lymphopenic potency of Caramel Colour III and THI has also been discussed in terms of reduced intestinal absorption of THI. A less potent protection from lymphopenia by PN administered intraperitoneally when compared to oral application was interpreted to indicate that PN may prevent the intestinal absorption of the lymphopenic constituent of Caramel Colour III (Conway and Paine, 1986b, Conway and Paine, 1988). However, a more recent study demonstrated that dietary PN can prevent the lymphopenic effect of intraperitoneally administered THI (Gobin and Paine, 1989). On what level vitamin B6 interferes with the lymphopenic potency of THI still remained unknown.

Aims of the studies presented in this thesis

The research presented in this thesis aimed to analyse effects of Caramel Colour III and THI on the immune system in more detail. In addition, functional consequences of the effects of Caramel Colour III and THI on the immune system, the mechanism of action of THI and the role of vitamin B6 were studied. This research was not only considered of importance for the benefit of risk assessment with respect to human intake of Caramel Colour III, but was also regarded important for basic immunological and immunotoxicological knowledge.

Chapter 2 of this thesis describes two studies carried out in order to characterize and compare effects of Caramel Colour III and THI on various lymphoid organs, and to study the influence of the dietary vitamin B6 content on these effects. Studies conducted in order to investigate the relevance of the observed changes in terms of functionality of the rat immune system are presented in Chapter 3. Chapter 4 is focussed on *in vitro* and *ex vivo* studies on Caramel Colour III and THI. In addition to previously reported studies, effects of Caramel Colour III and THI on the rat immune system were compared with effects of the vitamin B6 antagonist 4'-deoxypyridoxine (DOP) in more detail. The results of these studies are described and discussed in Chapter 5. In Chapters 6 and 7, studies conducted in mice and humans are presented. The pilot study in mice (Chapter 6) aimed to investigate whether effects of Caramel Colour III and THI in mice are similar to effects in rats. A study with human volunteers was regarded of importance because Class III Caramel Colours are very commonly used in many products for human consumption, some of which may contain quite high levels (Food Advisory Committee, 1987; Steering Group on Food Surveillance, 1987). Consumption of these products may result in an intake of Caramel Colour III higher than the current Acceptable Daily Intake (ADI) of 200 mg/kg body weight/day. It was questioned whether effects in (marginally vitamin B6 deficient) humans may occur upon dietary intake of Caramel Colour III. The intervention study presented in Chapter 7 was a first human study dealing with this question. The results of the research presented in the Chapters 2 to 7 of this thesis are summarized and generally discussed in Chapter 8.

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Chapter 2

Effects of the color additive Caramel Color III and 2-acetyl-4(5)-tetrahydroxybutyl-imidazole (THI) on the immune system of rats

Geert F. Houben [1,2,3], Henk van den Berg [2], Marga H. M. Kuijpers [2], Bert W. Lam [1], Henk van Loveren [3], Willem Seinen [1], and André H. Penninks [1,2]

1: Research Institute of Toxicology, Department of Immunotoxicology, University of Utrecht, Utrecht, The Netherlands

2: TNO-Toxicology and Nutrition Institute, Zeist, The Netherlands

3: National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands

Abstract

Administration of Ammonia Caramel Color (AC) to rats may decrease blood lymphocyte counts, especially in rats fed a diet low in vitamin B6. This effect is associated with 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI). To characterize and compare the effects of AC and THI and to study the influence of dietary pyridoxine, two studies in rats were conducted. Weanling rats fed a diet containing 2 to 3 ppm pyridoxine and exposed to 4% AC or 5.72 ppm THI in drinking water for 4 weeks showed reduced cell numbers in spleen and popliteal lymph nodes, as well as in the blood. Flow cytometric analyses demonstrated a comparable reduction in B and T lymphocytes. In blood, spleen and popliteal lymph nodes, $CD4^+$ lymphocytes were more reduced than $CD8^+$ cells. The number of bone marrow cells was not affected. Although thymus weight and cell number were not affected either, a decreased cortex over medulla area ratio and an increase in medullary cell density, largely due to an increase in $CD4^+$ thymocytes was observed. Decreased numbers of $ED2^+$ macrophages were observed in the thymic cortex and in the spleen red pulp. All effects observed were either less pronounced or absent in a study with rats fed a diet containing 11-12 ppm pyridoxine. The effects of AC and THI on the immune system were similar. Whereas AC-exposure was associated with changes in vitamin B6 status, THI did not induce obvious effects on vitamin B6 parameters. It is proposed that the effects of AC and THI on the immune system are not caused by a disturbance of vitamin B6 metabolism, but may in fact result from a disturbance of a specific pyridoxal 5'-phosphate-dependent process.

Introduction

Caramel color (E 150) refers to a large number of complex products, produced by the controlled heating of carbohydrates together with defined ranges of specified reactants. These dark-brown to black viscous liquids with the characteristic odour of burnt sugar and a somewhat bitter taste are among the oldest food colorants. Their use accounts for about 95% by weight of the permitted color additives used in food. Based on the reactants used for their production, caramel colors have been classified into four classes which differ in their chemical and functional properties as well as their applications (Ward, 1976; Noltes and Chappel, 1985; JECFA, 1986). Class III caramel colors constitute a class of caramel color very commonly used in many

products and their use accounts for 20 to 25% of the total use of caramel colors in the USA and for about 50% in Europe.

Administration of Caramel Color III, also known as Ammonia Caramel (AC), can cause a reduction in total white blood cell counts in rats (Evans *et al.*, 1977; Gaunt *et al.*, 1977; Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988) and in mice (Chapter 6; Iscaro *et al.*, 1988), due to reduced lymphocyte counts. This effect of AC in rats was found to depend on the vitamin B6 content of the diet. Addition of vitamin B6 to the diet of rats prevented the AC-induced reduction in the number of lymphocytes (Noltes and Chappel, 1985; Conway and Paine, 1988; Sinkeldam *et al.*, 1988; Gobin and Paine, 1989). Following the isolation and identification of 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI) from AC (Kröplien *et al.*, 1985), comparative studies indicated that this was the major, if not the only component of AC responsible for the reduction in the number of blood lymphocytes (Noltes and Chappel, 1985; Conway and Paine, 1986c; Conway and Paine, 1988; Sinkeldam *et al.*, 1988). Careful analysis of the effects of AC and THI and their mechanism of action is not only of importance for the benefit of risk assessment with respect to human intake of AC, but will also be important for basic immunological and immunotoxicological knowledge. In order to characterize and compare the effects of AC and THI on the various lymphoid organs and to study the influence of the dietary vitamin B6 content on these effects, two 4-week feeding studies with rats were conducted.

Materials and Methods

Materials. The specifications of the Ammonia Caramel (AC) used in these studies were as follows: Manufacturer: CPC, Manchester, UK; Batch: HT2207; K_{610} (absorbance at 610 nm, pathlength 1 cm, of an aqueous 0.1% w/v solution of AC): 0.18; % solids: 70.6; % N: 4.2; % S: 0.2; % ammoniacal N: 0.01; 4-methylimidazole content: 882 ppm; THI content:143 ppm. The 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)imidazole (THI), Lot MD-1, was synthesized according to the method described by Sweeny *et al.* (1985) and was kindly provided by the International Technical Caramel Association (ITCA, Washington, DC, USA).

Animals and maintenance. Young male Wistar-derived rats (RIV-TOX) from a specified-pathogen-free colony (RIVM, Bilthoven, The Netherlands) were housed in groups of four in wire-mesh floored cages in an animal room maintained at 23 ± 1°C,

with a light/dark cycle of 12 hr and a relative humidity of 50 ± 10%. The animals were fed a semi-purified casein diet (TNO, Zeist, The Netherlands) containing 2-3 (low) or 11-12 (high) ppm vitamin B6 (added as pyridoxine·HCl; Merck, Darmstadt, FRG). AC and THI were administered in the drinking water. Slightly acidified demineralized water (with HCl to pH 3.0) whether or not supplemented with AC or THI and the food were freely available.

Experimental design and conduct. Two studies were conducted: one with rats fed a diet containing 2-3, and one with a diet containing 11-12 ppm vitamin B6. In each study, groups of 12 rats were provided either with acidified demineralized water (controls), a 4% (w/w) AC solution (equal to a 5.72 ppm THI solution), or a 5.72 ppm (w/w) THI solution. The AC and THI exposure was started immediately after weaning the animals, and was continued during 28 or 29 days. Prior to the studies, the animals were allocated to treatment and control groups by computer randomization and proportionately to body weights. At the beginning of the studies, the rats had a body weight of 46.3 ± 5.7 g in the low and 49.7 ± 6.6 g in the high vitamin B6 diet study. During the experimental period, body weights of the individual rats and food intake on a cage basis were recorded weekly, and fluid intake was determined by weighing the water bottles every day. For practical reasons, the necropsy of the rats was spread over 2 days, viz. day 28 and 29. At days 28 and 29, 6 rats per group were killed. Results from AC- or THI-treated animals were compared to the controls of the same day. At the termination of the experiments, a blood sample was collected from the tip of the tail of all rats and blood smears were made. Subsequently, the animals were killed under ether anaesthesia by exsanguination from the abdominal aorta. From each animal, 2.5 ml blood was used to prepare serum and 5.0 ml was diluted with an equal volume of cooled (4°C) phosphate-buffered saline (PBS) containing 5 mg/ml ethylene-diaminetetraacetic acid-disodium salt (EDTA; BDH Chemicals Ltd., Pole, UK) in order to prevent coagulation. At necropsy, weights of thymus, spleen, liver, kidneys and adrenals were determined. In addition, femoral bone marrow, Peyer's patches as well as mesenteric, popliteal, axillary and inguinal lymph nodes were isolated. The tissues were processed for further examinations.

Determination of blood cell numbers. Samples of the diluted and decoagulated blood were used to determine total white and red blood cell numbers using a Coulter Counter model ZF (Coulter Electronics Ltd., Harpender, Herts, UK). An estimation of the relative numbers of the different types of leukocytes was obtained

by counting 200 cells in the blood smears after Pappenheim staining (Gorter and de Graaf, 1955). From the total and the differential leukocyte counts, the absolute numbers of the different types of leukocytes were calculated.

Determination of cell numbers of lymphoid organs. Cell suspensions in cooled (4°C) Dulbecco's PBS supplemented with 2 mM D-glucose (D(+)-glucose monohydrate, Merck, Darmstadt, FRG) (Dulbecco's PBS/gluc) were prepared from thymus, spleen and mesenteric and popliteal lymph nodes of 6 rats per group by mincing the organs and passing the tissue through a nylon sieve (pore diameter 250 μm). For the collection of bone marrow cells, the cavities of both femurs of 6 rats per group were flushed with Dulbecco's PBS/gluc using a syringe and the cells were passed through a nylon sieve. To remove cell clumps, all suspensions were drawn through a 25-gauge needle. Subsequently, the cells were washed, resuspended in Dulbecco's PBS/gluc and passed through a needle once more. Aliquots of the suspensions were used to determine the total number of nucleated cells using the Coulter Counter.

Flow cytometric analysis of lymphocyte subpopulations. Blood cells of 6 rats per group were enriched for lymphocytes by means of Ficoll-Paque centrifugation. Ficoll-Paque (3.0 ml; Pharmacia Fine Chemicals AB, Uppsala, Sweden) was injected under 4.5 ml of the diluted blood in a polycarbon tube. Separations were carried out in duplicate for 40 minutes at 400g and at 18°C. The interfaces of the duplicates were isolated and mixed. The enriched cells were washed twice and resuspended in Dulbecco's PBS/gluc. The cells isolated from blood, spleen, femoral bone marrow and mesenteric and popliteal lymph nodes were labeled by a direct immunofluorescence technique using the following mouse anti-rat monoclonal antibodies (mAb's) conjugated with fluorescein isothiocyanate (FITC): MARK1 (MARK1-FITC; Sanbio B.V., Uden, The Netherlands), recognizing nearly all peripheral B cells (Bazin *et al.*, 1984); OX19 (OX19-FITC; obtained from Dr. A. F. Williams, Oxford University, Oxford, UK), binding to $CD5^+$ T cells (Dallman *et al.*, 1984); OX8 (OX8-FITC; Dr. A. F. Williams, Oxford University, Oxford, UK), detecting $CD8^+$ T cells (Brideau *et al.*, 1980); ER2 (ER2-FITC; obtained from Dr. J. Rozing, TNO, Rijswijk, The Netherlands), detecting $CD4^+$ T cells (Joling *et al.*, 1985). The cells were incubated for 45 minutes at 4°C with 50 μl of FITC-conjugated mAb at an appropriate dilution in Dulbecco's PBS supplemented with 0.01% sodium azide (Dulbecco's PBS/NaN_3) as well as in the absence of mAb. The cells were washed, resuspended in 1 ml Dulbecco's PBS/NaN_3 supplemented with 0.1% paraformaldehyde and analysed on a fluorescence-activated cell sorter (FACS-

analyser, Beckton-Dickenson, Sunnyvale, Calif., USA) using the consort 30 analysis system (Beckton-Dickenson). At analysis, side scatter and forward scatter gates representing cell granularity and cell size, respectively, were set at the lymphocyte peak, eliminating monocytes and dead cells from the profiles. The fluorescence of the gated cells was displayed as a histogram and a cursor was set on basis of the fluorescence value of the unlabeled cells. Counts with a fluorescence level stronger than the background were considered positive.

Histology and immunohistochemistry. Liver, kidneys, adrenals, Peyer's patches, lymph nodes and parts of thymus and spleen of at least 6 rats per group were embedded in plastic after fixation in 4% phosphate-buffered formaldehyde. Sections of 2.5 μm stained with hematoxylin and eosin were examined by light microscopy. Peyer's patches, lymph nodes and parts of thymus and spleen embedded in Tissue-Tek were also snap-frozen in liquid nitrogen. Cryostat sections of 6-8 μm were air-dried and fixed in acetone. Staining was performed by subsequent incubation with an appropriate dilution of mouse anti-rat mAb's for 60 minutes and rabbit anti-mouse immunoglobulin conjugated to peroxidase (P161, Dakopatts, Copenhagen, Denmark) for 30 minutes. The sections were developed by incubation with 3-amino-9-ethylcarbazole using H_2O_2 as substrate. Examination was performed by light microscopy. A large panel of mouse anti-rat mAb's was used. Ab's used for staining of (subsets of) T cells and thymocytes in various stages of differentiation included ER2 (Joling *et al.*, 1985), ER4 (Joling *et al.*, 1985; Vaessen *et al.*, 1985), OX8 (Brideau *et al.*, 1980), OX19 (Dallman *et al.*, 1984), OX34 (Williams *et al.*, 1987; Clark *et al.*, 1988;), OX44 (Paterson *et al.*, 1987a; Paterson and Williams, 1987), HIS42 (Kampinga *et al.*, 1989), HIS44 (Kampinga and Aspinall, 1990) and HIS45 (Kampinga *et al.*, 1990), some of which have some extra specificities. In addition, Ab's directed against B cells (OX33, Woollett *et al.*, 1985; HIS14, Kroese *et al.*, 1985; MARK1, Bazin *et al.*, 1984), macrophages, interdigitating cells and dendritic cells (ED1, ED2 and ED3, Dijkstra *et al.*, 1985 and Beelen *et al.*, 1987; 1F119, Nagelkerken *et al.*, 1987), thymic epithelial cells (HIS37, HIS38 and HIS39, Kampinga *et al.*, 1987), MHC class-II antigens (HIS19, Stet *et al.*, 1985; OX6 and OX17, Fukumoto *et al.*, 1982), an Ab detecting the transferine receptor, OX26 (Jefferies *et al.*, 1985) and one directed against the IL2 receptor, OX39 (Paterson *et al.*, 1987b) were used. The ED mAb's were provided by Dr. C. D. Dijkstra (Free University, Amsterdam, The Netherlands), ER mAb's by Dr. J. Rozing (TNO, Rijswijk, The Netherlands), HIS mAb's by Dr. J. Kampinga (University of Groningen, Groningen, The Netherlands), OX mAb's by Dr. A. F.

Williams (Oxford University, Oxford, UK) and 1F119 by Dr. L. M. Nagelkerken (TNO, Rijswijk, The Netherlands). MARK1 was purchased from Sanbio B.V. (Uden, The Netherlands).

Determination of total IgM, IgG and IgA. Serum samples of 6 rats per group were stored at -20°C for total IgM, IgG and IgA analysis by enzyme-linked immunosorbent assay (ELISA)(Vos *et al.*, 1982) using goat anti-rat IgM (Nordic Pharmaceuticals and Diagnostics, Tilburg, The Netherlands), sheep anti-rat IgG (Vos *et al.*, 1982) and mouse anti-rat IgA mAb (van Loveren *et al.*, 1988). Horseradish peroxidase type VI, R.Z.3 (Sigma Chemicals Co., St. Louis, USA) was conjugated to the goat anti-rat IgM, sheep anti-rat IgG and to sheep anti-rat IgA (RIVM, Bilthoven, The Netherlands) according to Nakane and Kawaoi (1974). As substrate, 3,3',5,5'-tetramethylbenzidine (Sigma Chemicals Co., St. Louis, USA) was used. In each study, a serum pool of the control animals was used as reference serum.

Determination of vitamin B6 status. Decoagulated blood of 6 rats per group was centrifuged, and the diluted plasma was stored at -70°C. Livers of the same animals were frozen in liquid nitrogen and were also stored at -70°C. Pyridoxal kinase (PK) activity in liver homogenates was measured according to Chern and Beutler (1975). Plasma and tissue pyridoxal 5'-phosphate (PLP) contents were determined as described by Chabner and Livingston (1970). The tissue pyridoxamine 5'-phosphate (PMP) content was measured as PLP after nonenzymatic transamination of PMP with glyoxylic acid (Suelter and Snell, 1979). Plasma pyridoxal (PL) was determined according to Schrijver *et al.* (1981). The protein content in liver homogenates was determined by Coomassie Brilliant Blue-staining (Bio Rad protein assay; Bio Rad Reagents, Richmond, Calif., USA) using bovine serum albumin (Sigma Chemicals Co., St. Louis, USA) as reference.

Statistical analyses. Group means were analysed for significance of difference by the two-tailed Student's *t*-test. Data were considered significant when $p<0.05$.

Results

Food and water intake, body weight gain. No significant differences in water intake, food intake and body weight gain were observed between the tree groups in either of the studies (data not presented). The AC- and THI-treated rats were exposed to equivalent amounts of THI, gradually decreasing from a daily oral intake of about

TABLE 2.1

BODY WEIGHTS AND RELATIVE ORGAN WEIGHTS OF CONTROL AND AC- OR THI-EXPOSED RATS FED A DIET EITHER LOW OR HIGH IN VITAMIN B6 [a].

group	Body weight [b]	adrenals [c]	kidneys [c]	liver [c]	thymus [c]	spleen [c]
			low vitamin B6 diet			
control	187.1 ± 10.7	0.21 ± 0.04	7.57 ± 0.42	48.39 ± 2.72	4.06 ± 0.46	2.84 ± 0.36
AC	178.1 ± 14.4	0.23 ± 0.05	8.49 ± 0.34***	48.21 ± 2.63	3.88 ± 0.54	2.02 ± 0.30***
THI	176.6 ± 20.3	0.24 ± 0.04	7.80 ± 0.53	45.68 ± 4.10	3.83 ± 0.45	2.26 ± 0.49**
			high vitamin B6 diet			
control	185.1 ± 16.1	0.26 ± 0.04	7.76 ± 0.27	45.11 ± 2.48	3.83 ± 0.59	2.66 ± 0.07
AC	186.3 ± 19.0	0.25 ± 0.03	8.39 ± 0.60**	47.65 ± 2.61*	3.89 ± 0.42	2.44 ± 0.31
THI	192.7 ± 14.6	0.25 ± 0.05	7.78 ± 0.50	46.15 ± 2.77	3.93 ± 0.59	2.44 ± 0.22*

[a] Weanling rats fed a diet low (2-3 ppm) or high (11-12 ppm) in pyridoxine were exposed to 4% AC or to 5.72 ppm THI in drinking water for 4 weeks. The data are presented as mean ± SD of 12 rats per group and are expressed in g (b) or in g/kg body weight (c).

* $p<0.05$; ** $p<0.01$; *** $p<0.001$, two-tailed Student's *t*-test.

0.8 mg per kg body weight at the beginning of the study to about 0.5 mg per kg body weight at the end of the test period.

Terminal body weight and organ weights. Terminal body weights and relative organ weights as determined in both studies are presented in Table 2.1. No significant differences in terminal body weights and in the absolute and relative weights of adrenals, thymus and liver were observed, with the following exceptions: In THI-exposed rats in the low vitamin B6 diet study, a slight decrease in the absolute liver weight (to 89%; $p<0.05$) and in the absolute thymus weight (to 89%; $p<0.01$) was observed. In AC-treated rats in the high vitamin B6 diet study, the relative liver weights were slightly increased by 6%. Absolute kidney weights were increased by 8% ($p<0.05$) in AC-exposed rats in the high vitamin B6 diet study. Relative kidney weights of the AC-exposed rats were significantly increased by 12% in the low, and by 8% in the high vitamin B6 diet study. THI-exposure was not found to influence absolute or relative kidney weights. Absolute spleen weights of AC- and THI-exposed rats in the low vitamin B6 diet study were significantly decreased ($p<0.001$) to 67 and 75% of the controls, respectively. The corresponding relative weights of this organ were decreased to 71 and 80%, respectively. The mean relative spleen weight in the high vitamin B6 diet study was significantly decreased (to 92%) in THI-exposed rats.

Blood cell numbers and differential leukocyte counts. No differences were observed in blood monocyte, eosinophil and basophil numbers in either of the two studies (data not presented). Results of red and total white blood cell counts as well as blood neutrophil and lymphocyte numbers are presented in Table 2.2. Because blood cell numbers are subject to day to day variation, the data from days 28 and 29 are presented separately. No obvious effects of AC and THI on red blood cell numbers and on blood neutrophil counts were observed. Only in the case of the AC-treated rats in the high vitamin B6 diet study, dissected at day 28, a slight decrease in the red blood cell counts to 95% of the control numbers and an increase in the blood neutrophil numbers by 104% were noted. In the animals dissected at day 29, these differences were not observed. Total leukocyte numbers of AC- and THI-exposed rats were decreased to about 34% of the control counts in the low vitamin B6 diet study, due to a significant reduction in the number of blood lymphocytes to about 26%. In the high vitamin B6 diet study, a tendency of reduced blood leukocyte and lymphocyte numbers in AC- and THI-exposed rats was observed. This reached the level of statistical significance for the number of blood lymphocytes at day 28.

TABLE 2.2

RED AND WHITE BLOOD CELL COUNTS AS WELL AS BLOOD NEUTROPHIL AND LYMPHOCYTE NUMBERS OF CONTROL AND AC- OR THI-EXPOSED RATS FED A DIET EITHER LOW OR HIGH IN VITAMIN B6 [a].

	red blood cells		white blood cells		blood lymphocytes		blood neutrophils	
group	day 28	day 29	day 28	day 29	day 28	day 29	day 28	day 29
low vitamin B6 diet								
control	100 ± 9	100 ± 7	100 ± 30	100 ± 29	100 ± 31	100 ± 29	100 ± 44	100 ± 36
AC	95 ± 12	98 ± 7	28 ± 5***	34 ± 6***	22 ± 5***	28 ± 7***	112 ± 34	103 ± 27
THI	96 ± 8	97 ± 5	26 ± 9***	46 ± 11**	20 ± 8***	34 ± 9***	124 ± 25	158 ± 59
high vitamin B6 diet								
control	100 ± 5	100 ± 11	100 ± 18	100 ± 44	100 ± 17	100 ± 46	100 ± 72	100 ± 53
AC	95 ± 2*	100 ± 7	82 ± 16	76 ± 22	74 ± 14*	72 ± 25	204 ± 71*	121 ± 49
THI	95 ± 8	96 ± 9	68 ± 34	85 ± 44	63 ± 30*	79 ± 38	174 ± 126	180 ± 151

[a] Weanling rats fed a diet low (2-3 ppm) or high (11-12 ppm) in pyridoxine were exposed to 4% AC or to 5.72 ppm THI in drinking water for 4 weeks. The data are presented as mean ± SD of 6 rats per group and are expressed as % of the controls of the same day.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$, two-tailed Student's *t*-test.

TABLE 2.3

NUMBERS OF NUCLEATED CELLS ISOLATED FROM VARIOUS LYMPHOID ORGANS OF CONTROL AND AC- OR THI-EXPOSED RATS FED A DIET EITHER LOW OR HIGH IN VITAMIN B6 [a].

group	thymus [b]	spleen [c]	bone marrow [c]	popliteal LN [d]
		low vitamin B6 diet		
control	1.8 ± 0.2	7.9 ± 2.1	2.5 ± 0.4	6.4 ± 2.4
AC	1.4 ± 0.2**	3.7 ± 1.0**	2.2 ± 0.4	2.4 ± 1.7**
THI	1.7 ± 0.2	3.4 ± 0.5***	2.3 ± 0.2	2.5 ± 3.4*
		high vitamin B6 diet		
control	1.6 ± 0.2	6.5 ± 1.8	2.5 ± 0.4	4.3 ± 1.2
AC	1.5 ± 0.2	5.4 ± 0.9	2.2 ± 0.3	4.1 ± 1.6
THI	1.8 ± 0.4	4.4 ± 0.7*	2.4 ± 0.5	3.8 ± 1.1

[a] Weanling rats fed a diet low (2-3 ppm) or high (11-12 ppm) in pyridoxine were exposed to 4% AC or to 5.72 ppm THI in drinking water for 4 weeks. The data are presented as mean ± SD of 6 rats per group and are expressed in 10^9 (b), 10^8 (c) or 10^6 (d) nucleated cells per organ. Numbers of bone marrow and popliteal lymph node (LN) cells indicate the total number of nucleated cells isolated from both popliteal lymph nodes and femoral bones.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$, two-tailed Student's *t*-test.

Cell numbers of lymphoid organs. Total numbers of nucleated cells isolated from lymphoid organs are presented in Table 2.3. Numbers of bone marrow cells did not differ in AC- or THI-exposed rats when compared to the corresponding controls in either of the two studies. Thymus cell numbers in the low vitamin B6 diet study were decreased to 78% of the controls in AC-treated rats. Total numbers of spleen cells in the low vitamin B6 diet study were decreased to 47 and 43% of control counts in AC- and THI-exposed rats, respectively. In the high vitamin B6 diet study, a reduction in spleen cell numbers to 68% was observed in THI-treated rats. Cell numbers isolated from popliteal lymph nodes in the low vitamin B6 diet study were decreased to 38 and 39% in AC- and THI-exposed rats, respectively.

TABLE 2.4

RESULTS OF FLOW CYTOMETRIC ANALYSIS OF LYMPHOCYTE SUBPOPULATIONS IN VARIOUS LYMPHOID ORGANS OF CONTROL AND AC- OR THI-EXPOSED RATS FED A DIET LOW IN VITAMIN B6 [a].

group	T cells	B cells	Th cells	Tsup/cyt	B:T	Th:Tsup/cyt
			blood			
control	62.0 ± 6.7(5)	26.3 ± 5.7	49.1 ± 14.5	42.9 ± 4.6	0.45 ± 0.10(5)	1.14 ± 0.31
AC	53.0 ± 7.2	23.1 ± 4.8	12.9 ± 3.3***	43.3 ± 7.6	0.44 ± 0.12	0.27 ± 0.09***
THI	58.4 ± 7.9	21.9 ± 5.7	23.5 ± 7.7**	45.2 ± 6.0	0.39 ± 0.18	0.40 ± 0.10***
			spleen			
control	54.7 ± 8.1	35.5 ± 5.8	23.8 ± 3.6	51.7 ± 8.3	0.67 ± 0.18	0.47 ± 0.08
AC	53.4 ± 4.6	33.2 ± 5.7	16.2 ± 2.0**	54.7 ± 8.8	0.63 ± 0.12	0.31 ± 0.08**
THI	55.6 ± 5.3	32.8 ± 10.4	17.8 ± 3.6*	58.0 ± 6.9	0.61 ± 0.23	0.31 ± 0.08**
			popliteal lymph nodes			
control	68.5 ± 4.7	20.3 ± 5.5	35.4 ± 5.4	47.9 ± 8.9	0.30 ± 0.08	0.75 ± 0.13
AC	76.8 ± 7.9	19.4 ± 7.5	23.6 ± 8.4*	50.7 ± 24.4	0.26 ± 0.10	0.52 ± 0.13*
THI(5)	72.0 ± 14.0	18.2 ± 13.3	18.6 ± 11.2**	47.0 ± 26.7	0.29 ± 0.29	0.45 ± 0.22*

[a] Weanling rats fed a diet low (2-3 ppm) in pyridoxine were exposed to 4% AC or to 5.72 ppm THI in drinking water for 4 weeks. Unless indicated (.), data are presented as mean ± SD of 6 rats per group and are expressed as % positive cells (T cells, B cells, Th cells and Tsup/cyt cells) and as B over T and Th over Tsup/cyt ratios. See text for used mAb's.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$, two-tailed Student's *t*-test.

Lymphocyte subset numbers. Results of lymphocyte subpopulation analyses in blood, spleen and popliteal lymph nodes in the low vitamin B6 diet study are shown in Table 2.4. The percentages of $MARK1^+$ and $OX19^+$ cells as well as the $MARK1^+/OX19^+$ ratios were not changed. However, exposure to AC or THI in the low vitamin B6 diet study induced a reduction in the percentage of $ER2^+$ cells in these tissues. Since the percentage of $OX8^+$ cells was not affected, a decrease in the $ER2^+/OX8^+$ ratios was observed. In the high vitamin B6 diet study (flow cytometric analysis data not presented), a clear tendency of reduced percentages of $ER2^+$ cells was observed in blood and spleen of AC- and THI-treated rats. This reduction resulted in a significant ($p<0.01$) decrease in the $ER2^+/OX8^+$ ratios in spleens of AC- and THI-exposed rats (80 and 77% of the controls, respectively). With the exception of an increase in the percentage of $OX8^+$ cells in popliteal lymph nodes of AC-exposed rats (by 29%; $p<0.05$), no other significant differences were observed in lymphocyte subset distributions in blood, spleen and popliteal lymph nodes in the high vitamin B6 diet study. No effects were observed on lymphocyte subset distribution in mesenteric lymph nodes and in bone marrow in either of the two studies (data not presented).

Histological observations. No histopathologic abnormalities were observed in kidneys, adrenals and livers of AC- or THI-exposed rats in either of the two studies. In thymi of AC- and THI-exposed rats in the low vitamin B6 diet study, however, serial sections stained with hematoxylin and eosin demonstrated a decrease in cortical area, whereas the medullary area appeared to be enlarged. Moreover, an increased cell density was observed in the medullas (Figure 2.1). By means of immunohistochemical staining, it was demonstrated that the increase in medullary area and cell density was caused, in particular, by a strong increase in the number of medullary $CD4^+$ ($ER2^+$), $OX44^+$ and $HIS45^+$ thymocytes (Figure 2.2). Staining with mAb's detecting B cells did not demonstrate a change in the number or localisation of B cells in the thymus. Investigations in the thymi using most other mAb's only confirmed the decrease in thymic cortex/medulla area ratio but did not provide additional information, with one exception. Staining with the mAb ED2 demonstrated a very strong decrease in the number of cortical macrophages positive for the differentiation antigen recognized by this mAb. Results from staining with the general macrophage marker ED1 indicated that the number of macrophages was not obviously changed. A comparable decrease in the expression of the antigen

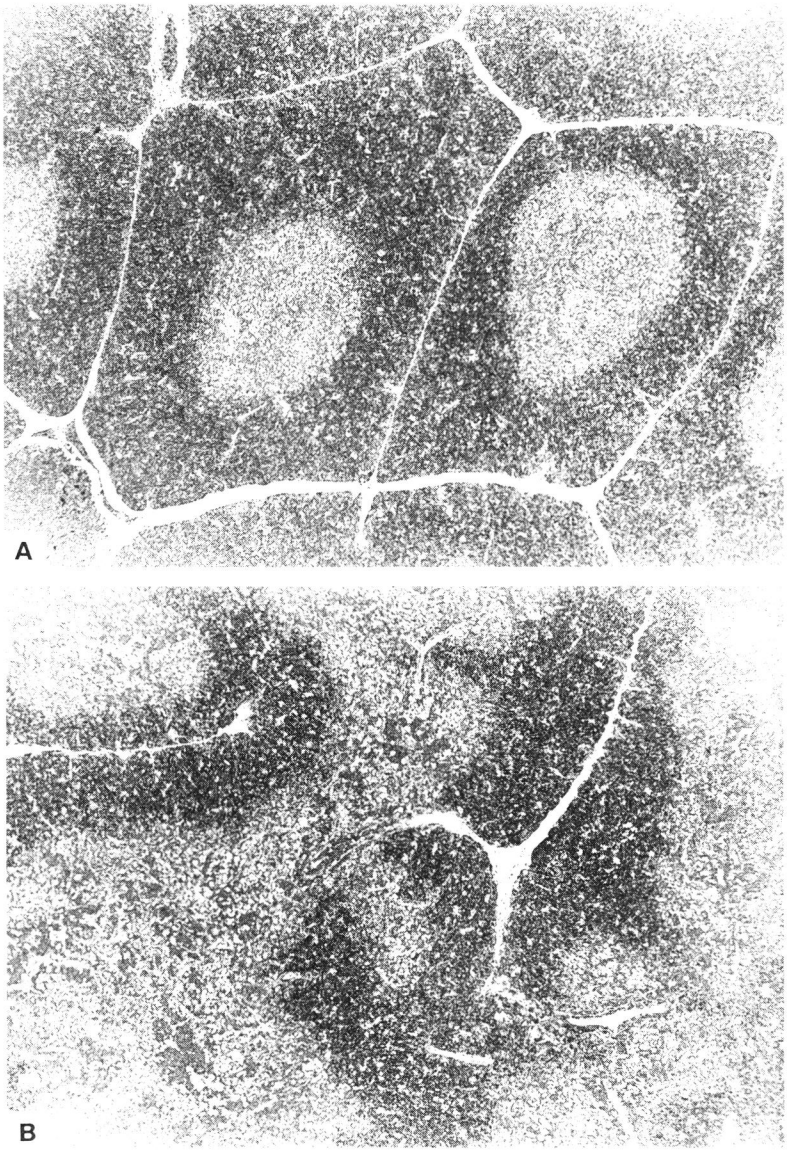

Figure 2.1: Thymus section from a control (A) and a THI-exposed (B) rat. Weanling rats were fed a diet with a low (2-3 ppm) content of vitamin B6. The THI-exposed rat was exposed to 5.72 ppm THI in drinking water for 4 weeks. Stained with hematoxylin and eosin. Note the decrease in cortical and the increase in medullary area.

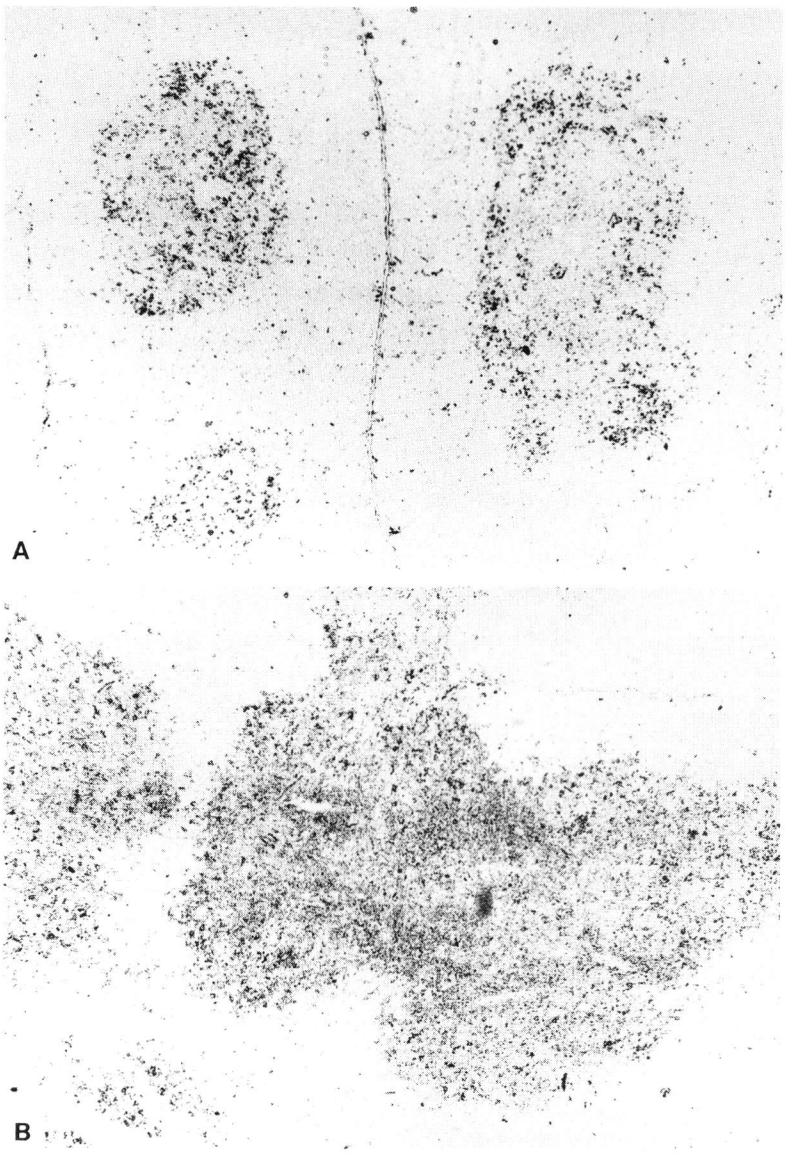

Figure 2.2: Thymus section from a control (A) and a THI-exposed (B) rat. Weanling rats were fed a diet with a low (2-3 ppm) content of vitamin B6. The THI-exposed rat was exposed to 5.72 ppm THI in drinking water for 4 weeks. Stained immunohistochemically with OX44. Note the increased density of $OX44^+$ cells in the medulla.

recognised by ED2 was observed in the red pulp of spleens of AC- and THI-treated rats in the low vitamin B6 diet study. In addition, a decrease in the number of $ER4^+$ cells was observed in spleens of these rats. Pathologic examination of hematoxylin and eosin stained spleen sections of these animals demonstrated an increase in the number of megakaryocytes in the red pulp. Examination of lymph nodes of AC- and THI-exposed rats in the low vitamin B6 diet study showed a reduced cellularity. None of the effects of AC and THI such as found by (immuno)histological examinations were observed in the high vitamin B6 diet study. Beside those described, no other abnormalities were observed.

Total IgM, IgG and IgA levels. Results of total serum IgM, IgG and IgA determinations are listed in Table 2.5. No significant differences in total IgM and IgG levels were observed in either of the two studies. Total IgA levels in AC-exposed rats in the high vitamin B6 diet study were decreased to 49% of control levels.

TABLE 2.5

TOTAL IGM, IGG AND IGA LEVELS IN SERUM OF CONTROL AND AC- OR THI-EXPOSED RATS FED A DIET EITHER LOW OR HIGH IN VITAMIN B6 [a].

group	IgM	IgG	IgA
	low vitamin B6 diet		
control	100 ± 37	100 ± 33	100 ± 69
AC	83 ± 14	86 ± 44	142 ± 134
THI	80 ± 23	76 ± 34	94 ± 87
	high vitamin B6 diet		
control	100 ± 34	100 ± 35	100 ± 30
AC	85 ± 4	90 ± 24	49 ± 35*
THI	100 ± 35	99 ± 37	66 ± 67

[a] Weanling rats fed a diet low (2-3 ppm) or high (11-12 ppm) in pyridoxine were exposed to 4% AC or to 5.72 ppm THI in drinking water for 4 weeks. The data are presented as mean ± SD of 6 rats per group and are expressed in % of the controls.

* $p<0.05$, two-tailed Student's *t*-test.

Parameters of the vitamin B6 status. Results of the determination of the various parameters of the vitamin B6 status are presented in Table 2.6. In the low vitamin B6 diet study, plasma pyridoxal (PL) and pyridoxal 5'-phosphate (PLP) as well as liver pyridoxamine 5'-phosphate (PMP) levels were decreased in AC-exposed rats, whereas treatment with AC in the high vitamin B6 diet study was associated with decreased levels of PLP and PMP in the liver. Plasma PL/PLP and liver PLP/PMP ratios were not affected by AC-exposure in either of the studies. The activity of liver pyridoxal kinase was not affected by exposure to AC. In the low vitamin B6 diet study, liver PLP/PMP ratios in THI-exposed rats appeared to be increased. There were no other significant differences in any of the parameters associated with exposure to THI in either of the two studies.

Discussion

The results obtained in these studies indicate that an oral intake of a 4% AC solution (AC batch containing 143 mg THI/kg ; AC manufacturers currently meet the proposed specifications of the EC and limit THI to no more than 25 mg/kg AC) or a solution of 5.72 ppm THI by male Wistar rats fed a diet low in vitamin B6 (2-3 mg/kg) causes a strong and comparable decrease in total white blood cell counts, due to reduced lymphocyte counts. In addition, reduced numbers of nucleated cells were observed in spleen and popliteal lymph nodes, whereas absolute and relative spleen weights were also decreased. The reduced cell numbers in blood, spleen and popliteal lymph nodes were caused by a comparable decrease in the number of T and B cells, whereas within the T cell population, $CD4^+$ T lymphocytes were slightly more reduced than $CD8^+$ T cells. Reduced leukocyte and blood lymphocyte counts in rats have been reported from several studies with AC (Evans *et al.*, 1977; Gaunt *et al.*, 1977; Conway and Paine, 1986a,b,c; Conway and Paine, 1988; Sinkeldam *et al.*, 1988; Gobin and Paine, 1989) and THI (Conway and Paine, 1986c; Conway and Paine, 1988; Sinkeldam *et al.*, 1988; Gobin *et al.*, 1989; Gobin and Paine, 1989), whereas similar observations on blood lymphocyte subset distribution in THI-exposed rats were reported by Gobin *et al.* (1989). Reduced absolute (Gaunt *et al.*, 1977) and relative (Sinkeldam *et al.*, 1988) spleen weights have been reported as well. Relative thymus weights were not found to be changed after exposure to AC or THI, which is in accordance with observations from several short-term studies with AC- and THI-exposure for less then 4 weeks (Sinkeldam *et al.*, 1988). However, results on

TABLE 2.6

PLASMA PL AND PLP AND LIVER PLP AND PMP LEVELS AND PK ACTIVITY IN LIVER OF CONTROL AND AC- OR THI-EXPOSED RATS FED A DIET EITHER LOW OR HIGH IN VITAMIN B6 [a].

	low vitamin B6 diet			high vitamin B6 diet		
parameter	control	AC	THI	control	AC	THI
plasma PL [b]	36 ± 7	27 ± 5*	36 ± 8	234 ± 47	215 ± 27	255 ± 89
plasma PLP [b]	86 ± 18	58 ± 7**	80 ± 18	299 ± 62	266 ± 51	335 ± 69
PL/PLP [c]	0.42 ± 0.09	0.47 ± 0.08	0.45 ± 0.05	0.79 ± 0.09	0.83 ± 0.15	0.76 ± 0.19
liver PLP [d]	8.9 ± 0.7	8.2 ± 0.9	9.7 ± 1.2	11.4 ± 1.3	9.1 ± 1.1**	10.7 ± 0.6
liver PMP [d]	13.6 ± 1.2	12.0 ± 1.0*	12.8 ± 1.8	16.6 ± 2.2	13.4 ± 1.4*	15.6 ± 1.4
PLP/PMP [c]	0.65 ± 0.05	0.68 ± 0.06	0.76 ± 0.05**	0.71 ± 0.17	0.68 ± 0.06	0.69 ± 0.07
PK/protein [e]	4.9 ± 0.9	4.3 ± 1.0	5.5 ± 1.4	5.3 ± 1.4	4.6 ± 0.8	5.6 ± 1.2

[a] Weanling rats fed a diet low (2-3 ppm) or high (11-12 ppm) in pyridoxine were exposed to 4% AC or to 5.72 ppm THI in drinking water for 4 weeks. The data are presented as mean ± SD of 6 rats per group and are expressed in nmol/l (b), as ratio (c), in nmol/g liver (d) or in nmol/h x mg protein (e). PL: pyridoxal; PLP: pyridoxal 5'-phosphate; PMP: pyridoxamine 5'-phosphate; PK: pyridoxal kinase.

* $p<0.05$; ** $p<0.01$, two-tailed Student's *t*-test.

relative thymus weights reported from a 13-week study demonstrated a reduction after exposure to AC (Gaunt *et al.*, 1977). The number of blood neutrophils was not markedly affected by AC or THI. This also is in accordance with several studies on AC (Sinkeldam *et al.*, 1988) and THI (Gobin *et al.*, 1989), although increased numbers of neutrophils have been observed in other studies with AC (Evans *et al.*, 1977; Gaunt *et al.*, 1977; Houben *et al.*, unpublished results) and THI (Chapter 5; Sinkeldam *et al.*, 1988). Interestingly, increased numbers of blood neutrophils in rats fed a diet low in vitamin B6 may also be induced by the vitamin B6 antagonist 4'-deoxypyridoxine (DOP) (Robson and Schwarz, 1975; Houben *et al.*, unpublished results). The lymphopenic activity of AC and THI has been compared with that of DOP by several authors (Schreurs and Sinkeldam, 1985; Conway and Paine, 1986a; Sinkeldam *et al.*, 1988).

At (immuno)histopathological examination, lymphocyte depletion was observed in peripheral lymphoid organs, reflecting the observed reduced nucleated cell counts in these organs. In thymus, a decrease in cortical area and an increase in medullary area and cell density were observed. Although the number of $CD8^+$ cells was also increased, the increase in medullary cell density was largely due to a pronounced increase in the number of $CD4^+$ cells. Whether this stronger increase in $CD4^+$ thymocytes is related to the more pronounced depletion of peripheral $CD4^+$ cells remains to be determined. Since it is known that in the rat spleen, the differentiation antigen recognized by ER4 is not present on mature T-cells (Vaessen *et al.*, 1985), the observed decrease in the number of $ER4^+$ cells in spleens of AC- and THI-exposed rats possibly indicates a reduced migration of cells from thymus.

Although a vitamin B6 content of 6 to 7 ppm is generally accepted as an adequate level in rat diet (National Academy of Sciences, 1978), a diet containing 2 to 3 ppm pyridoxine is not considered deficient for rats on basis of growth (Driskell *et al.*, 1973; van den Berg *et al.*, 1982) and behavioral patterns (Driskell *et al.*, 1973). All effects of AC and THI were either less pronounced or absent in rats fed a diet high in pyridoxine. However, a tendency of reduced blood leukocyte and lymphocyte numbers was still observed upon AC- or THI-exposure of rats fed the diet containing 11-12 ppm vitamin B6, reaching the level of statistical significance for the number of blood lymphocytes at day 28. Moreover, a decrease in the $CD4^+/CD8^+$ ratios in spleen was still apparent. With respect to AC- and THI-induced lymphopenia, a protective effect of dietary pyridoxine was reported from studies with AC (Conway and Paine, 1986b; Conway and Paine, 1988; Sinkeldam *et al.*, 1988; Gobin and Paine,

1989) and THI (Gobin and Paine, 1989). Results of total IgA determinations demonstrated a significant decrease (to 49%) in AC-exposed rats in the high vitamin B6 diet study. Because the Wistar strain of rats appeared to include high and low IgA-responders, the data were also analysed after a correction was applied to exclude extreme values using the Dixon test for outlier detection (Dixon, 1950). After correction, IgA levels in THI-exposed rats indicated to be decreased to 80% ($p<0.05$) in the low, and to 41% ($p<0.05$) of the control levels in the high vitamin B6 diet study. Since all effects of AC and THI on the immune system studied so far are less pronounced when the pyridoxine content of the diet is increased, the results on IgA levels suggest a remarkable exception in this respect and require further investigations.

Relative kidney weights were increased in AC- exposed rats in both studies. Increased relative kidney weights in AC-exposed rats were also reported by Gaunt *et al.* (1977). Since no significant differences in the effects of AC and THI on the immune system have been observed and THI does not influence relative kidney weights, it is unlikely that the effect of AC on the kidney weight is related to the immunotoxicity. Morphological examination of the kidneys did not reveal any pathological effect.

In the past few years, studies conducted in an attempt to elucidate the mechanism responsible for the lymphopenic action of AC and THI concentrated on the role of vitamin B6 (Spector and Huntoon, 1982; Schreurs and Sinkeldam, 1985; Conway and Paine, 1986a,b; Conway and Paine, 1988; Sinkeldam *et al.*, 1988; Gobin and Paine, 1989). However, not much progress has been made in this respect. Because there is some evidence indicating that AC affects mammalian vitamin B6 metabolism (Spector and Huntoon, 1982; Schreurs and Sinkeldam, 1985; Sinkeldam *et al.*, 1988), various parameters of the vitamin B6 status were determined. The results indicate that exposure to AC may be associated with decreased levels of plasma pyridoxal (PL) and pyridoxal 5'-phosphate (PLP) and liver PLP and pyridoxamine 5'-phosphate (PMP). Decreased plasma PLP levels in AC-exposed rats have been reported before (Schreurs and Sinkeldam, 1985; Sinkeldam *et al.*, 1988). Although it was demonstrated that AC may contain a competitive inhibitor of pyridoxal kinase (PK) (Spector and Huntoon, 1982; Schreurs and Sinkeldam, 1985), the *in vivo* studies reported here did not demonstrate a significant effect of AC on maximum PK activity in the liver. Exposure to THI was not found to be associated with obvious changes in the vitamin B6 status, as reported before (Sinkeldam *et al.*, 1988). Therefore, it is

unlikely that the lymphopenia induced by AC and THI is caused by an effect on vitamin B6 metabolism. Moreover, AC-induced lymphopenia is not always accompanied by changes in the vitamin B6 status either (Conway and Paine, 1986a; Houben *et al.*, unpublished results). Data indicating that the PK inhibiting compound in AC (Spector and Huntoon, 1982) has a molecular weight different from that of THI (Kröplien *et al.*, 1985), 360 vs 230, respectively, indicate that the effects of AC on vitamin B6 metabolism are caused by an other compound.

On what level vitamin B6 interferes with the lymphopenic potency of THI still remains unknown. However, studies in rats with the lymphopenic vitamin B6 antagonist DOP demonstrated a decrease in the number of lymphocytes that was apparent within 3 days (Schreurs and Sinkeldam, 1985; Sinkeldam *et al.*, 1988), indicating a time-effect relationship comparable to that of AC and THI (Chapter 5; Sinkeldam *et al.*, 1988). Lymphocyte counts may also be decreased by feeding a vitamin B6 deficient diet with a pyridoxine content of 1 ppm or below. This effect appears to be maximal only after 3 months (Schreurs and Sinkeldam, 1985). Classical signs of a vitamin B6 deficiency have never been observed in studies with AC or THI. Considering the effects of DOP and vitamin B6 deficiency on the number of blood lymphocytes, the influence of vitamin B6 on the AC- and THI-induced effects, together with the rapid onset and the selectivity of the effect of THI, we propose that THI disturbs a specific PLP-dependent process. This process does not necessarily involve lymphocytes. Specifically, the observations from immunohistochemical studies indicating a remarkable decrease in the number of macrophages positive for the mAb ED2 in AC- and THI-exposed rats may be of importance. ED2 is considered to recognize a membrane antigen (Dijkstra *et al.*, 1985) on well-differentiated macrophages, as indicated by a higher expression in a steady state population of peritoneal macrophages in comparison to inflammatory macrophages (Beelen *et al.*, 1987; Bos *et al.*, 1989). Although the expression of the antigen recognised by ED2 has not yet been clearly associated with functional characteristics of the cells, it has been suggested that $ED2^+$ macrophages play a role in constituting an immunoproliferative microenvironment in peripheral lymphoid tissues (Matsuno *et al.*, 1989) and in the regulation of proliferation and maturation of cortical thymocytes (Sminia *et al.*, 1986). Moreover, the observed preferential localisation of $ED2^+$ macrophages in the vicinity of plasma cells in various lymphoid tissues (Dijkstra *et al.*, 1985; van der Brugge-Gamelkoorn *et al.*, 1985; Sminia and Jeurissen, 1986) suggests a role of $ED2^+$ macrophages in plasma cell function. Therefore, a change in

activity and differentiation of the total population of macrophages, as possibly indicated by a decrease in the number of $ED2^+$ macrophages and supported by observations from immune function studies demonstrating an increased capacity of AC-exposed rats to clear *Listeria monocytogenes* (Chapter 3), is likely to be associated with a disturbance in immunoproliferative processes and with a disturbed regulation of proliferation and maturation of thymocytes and may explain the observed *in vivo* effects of AC and THI.

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Chapter 3

Immunotoxic effects of the color additive Caramel Color III and 2-acetyl-4(5)-tetrahydroxybutyl-imidazole (THI); immune function studies in rats

Geert F. Houben [1,2,3], André H. Penninks [2,3], Willem Seinen [2], Joseph G. Vos [1], and Henk van Loveren [1]

1: *National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands*

2: *Research Institute of Toxicology, Department of Immunotoxicology, University of Utrecht, Utrecht, The Netherlands*

3: *TNO Toxicology and Nutrition Institute, Zeist, The Netherlands*

Abstract

Administration of the color additive Caramel Color III (AC) may cause a reduction in total white blood cell counts in rats, due to reduced lymphocyte counts. Beside lymphopenia, several other effects in rat have been described. The effects are caused by the imidazole derivative 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI) and occur especially in rats fed a diet low in vitamin B6. In the present paper, several immune function studies on AC and THI with rats fed a diet low, but not deficient in vitamin B6 are presented and discussed. Rats were exposed to 0.4 or 4% AC or to 5.72 ppm THI in drinking water during, and for 28 days prior to the start of immune function assays. Resistance to *Trichinella spiralis* was examined in an oral infection model and clearance of *Listeria monocytogenes* upon an intravenous infection was studied. In addition, natural cell-mediated cytotoxicity of splenic and non-adherent peritoneal cells and the antibody response to sheep red blood cells were studied. From the results it is concluded that exposure of rats to AC or THI influenced various immune function parameters. Thymus-dependent immunity was suppressed, whereas parameters of the non-specific resistance were also affected, as shown by a decreased natural cell-mediated cytotoxicity in the spleen and an enhanced clearance of *Listeria monocytogenes*.

Introduction

Caramel color III, also known as ammonia caramel color (AC), is commonly used as a color additive in many products for human consumption. Administration of AC may cause a reduction in blood lymphocyte counts in rats (Evans *et al.*, 1977; Gaunt *et al.*, 1977; Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988; Chapter 2) and in mice (Iscaro *et al.*, 1988; Chapter 6). Beside lymphopenia, several other effects in rat have been described recently (Chapter 2), among which a reduced cell number in spleen and popliteal lymph nodes, a decreased CD4+/CD8+ lymphocyte ratio in blood, spleen and popliteal lymph nodes, a decreased cortex over medulla area ratio and an increase in medullary cell density in thymus, and a reduced number of $ED2^+$ macrophages in the thymic cortex and in the spleen red pulp. The effects are caused by the imidazole derivative 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI) (Kröplien *et al.*, 1985; Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988; Chapter 2)

and occur especially in rats fed a diet low in vitamin B6 (Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988; Gobin and Paine, 1989; Chapter 2).

Studies on the functionality of the immune system of AC- or THI-exposed animals have not been reported yet. Information on immune functions was considered essential in order to evaluate the significance of the reported AC- and THI-induced effects on the rat immune system. Moreover, immune function assessment may help to clarify the mechanism of action of THI. Therefore, several immune function studies on AC and THI were conducted with rats fed a diet low, but not deficient in vitamin B6. The resistance to *Trichinella spiralis* was examined by determination of serum immunoglobulin levels and by the yield of muscle larvae in an oral infection model. The clearance of *Listeria monocytogenes* upon an intravenous infection and the natural cell-mediated cytotoxicity of splenic and peritoneal cells were studied. Finally, the antibody response to sheep red blood cells was investigated. The results of these studies are presented and discussed.

Materials and Methods

Materials. Caramel Color III (AC) used in these studies was manufactured by CPC (Manchester, UK) and was a gift of the British Caramel Manufacturers Association (BCMA, London, UK). The specifications of the batch of AC were as follows: Batch: HT 2207; K_{610} (absorbance at 610 nm, pathlength 1 cm, of an aqueous 0.1% w/v solution of AC): 0.18; % solids: 70.6; % N: 4.2; % S: 0.2; % ammoniacal N: 0.01; 4-methylimidazole content: 882 ppm; THI content:143 ppm. It should be recognized that the AC used in these studies was prepared for research purposes using modified processing conditions. The THI content exceeds the limit defined for commercial Caramel Color III. The 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)imidazole (THI), Lot MD-1, was synthesized according to the method described by Sweeny (Sweeny *et al.*, 1985) and was kindly provided by the International Technical Caramel Association (ITCA, Washington, DC, USA).

Animals and maintenance. For the infection models and the natural cell-mediated cytotoxicity studies, SPF-derived random-bred weanling male Wistar rats (RIV-TOX, RIVM, Bilthoven, The Netherlands) weighing 52.3 ± 10.9 g were housed in stainless-steel wire cages, two rats per cage. The antibody response to sheep red blood cells was studied in random-bred young male Wistar rats obtained from Harlan CPB (Zeist, The Netherlands), housed in groups of four in plastic cages on a bedding of

wood chips. All animals were kept at a temperature of 22 ± 2°C with a relative humidity of 45 to 65%, and a 12-hr light/dark cycle. Semi-purified casein diet (TNO Toxicology and Nutrition Institute, Zeist, The Netherlands) containing 2 to 3 ppm vitamin B6 (supplemented as pyridoxine·HCl, Merck, Darmstadt, FRG) and slightly acidified (with HCl to pH 3.0) demineralized drinking water whether or not supplemented with AC or THI were provided *ad libitum*. During the studies, individual body weights as well as food and fluid intake on a cage basis were recorded until start of the immune function tests.

Experimental design. For examination of effects on immune function parameters using the infection models (resistance to *Trichinella spiralis* and clearance of *Listeria monocytogenes*) and the natural cell-mediated cytotoxicity assays, weanling rats in a first study were exposed to 0.4 or 4% AC in drinking water. The clearance of *L. monocytogenes* was reinvestigated in a second study with equivalent AC-exposures. To study the immune response upon immunization with sheep red blood cells (SRBC), rats were exposed to 5.72 ppm THI in drinking water (5.72 ppm THI is equivalent to the 4% AC solution used in the other studies). Control rats received no AC or THI. The animals were randomly allocated to treatment and control groups. The immune function tests were all started at day 28 of exposure. During the tests, exposure to AC or THI was continued.

Determination of white blood cell and blood lymphocyte numbers. At day 15 of exposure in the first study, blood samples were taken from the orbital plexus of 10 rats per group under light ether anesthesia. The blood was collected in heparinized tubes and the samples were used for determination of white blood cell numbers using a Coulter Counter model ZF (Coulter Electronics Ltd., Harpender, Herts, UK). At day 28 of exposure in the second study of the clearance of *L. monocytogenes*, blood samples from 10 animals per group taken prior to infection were used for the determination of white blood cell count and lymphocyte numbers using a Sysmex K-1000 Hemoanalyser (Toa Medical Electronics Co. Ltd, Japan).

Resistance to Trichinella spiralis infection. After 28 days of exposure, 12 rats per group were infected orally with 1000 *T. spiralis* larvae. The strain of *T. spiralis* used and its maintenance were as described previously (Ruitenberg *et al.*, 1977). Muscle larvae for the infection were obtained from infected Swiss mice after conventional digestion of the carcass with HCl and pepsin (Köhler and Ruitenberg, 1974). To reduce interference with the infectivity of the larvae, digestion was not prolonged beyond 2 hr. To investigate the resistance to the *T. spiralis* infection, the yield of

muscle larvae in eviscerated and skinned animals was determined by the digestion method (Köhler and Ruitenberg, 1974) at day 42 after infection. The class-specific anti-*T. spiralis* antibody response was determined using sera obtained 14 and 42 days after infection. The specific immunoglobulin titers were determined by ELISA (Ruitenberg *et al.*, 1975) using sheep anti-rat IgG (Vos et al., 1982) and goat anti-rat IgM (Nordic Pharmaceuticals and Diagnostics, Tilburg, The Netherlands), conjugated to horseradish peroxidase type VI, R.Z.3 (Sigma Chemicals Co., St. Louis, USA) according to the method of Nakane and Kawaoi (Nakane and Kawaoi, 1974). Specific IgE and IgA titers were determined using monoclonal mouse anti-rat IgE (MARE-1, Sanbio B.V., Uden, The Netherlands) and monoclonal mouse anti-rat IgA (van Loveren *et al.*, 1988). Peroxidase-conjugated affinity purified rat anti-mouse IgG (H+L) (Pelfreeze, Arkansis, USA) was applied as peroxidase-labeled conjugate. As substrate, 3,3',5,5'-tetramethylbenzidine (Sigma Chemicals Co., St. Louis, USA) was used. A pooled preserum was used as negative control. The pooled preserum was measured at a 1:4 dilution. The average extinction in negative control wells, to which twice the standard deviation was added, provided the reference value taken to determine the titer in the test sera. Each serum was titrated starting at a 1:4 dilution and the reciprocal of the furthest serum dilution giving a positive ELISA, i.e. an extinction higher than the reference value, was read as the titer. In the calculation of the results, sera that were negative at a dilution of 1:4 were considered positive at a 1:2 dilution.

Clearance of Listeria monocytogenes. L. monocytogenes (strain L242/73 type 4b) was cultured as described by Ruitenberg and Van Noorle Jansen (1975). After 28 days of exposure, rats were injected intravenously with $5x10^5$ *L. monocytogenes* bacteria. The clearance of *L. monocytogenes* was investigated by determination of the number of bacteria in the spleen at day 2 (study 1), or at days 2, 5, 8, and 14 (study 2) after infection. To establish this, 12 (study 1) or 10 (study 2) rats per group were killed on the respective days after infection and spleens were isolated and homogenised. Serial dilutions were plated and the viable counts of *L. monocytogenes* were determined as described by Ruitenberg and Van Noorle Jansen (1975). In the calculation of the results, *Listeria* counts below 10^2 per g organ were computed as 10^2.

Natural cell-mediated cytotoxicity of spleen and peritoneal cells. After 28 days of exposure, natural cell-mediated cytotoxicity of spleen and peritoneal cells was studied *ex vivo* by using a ^{51}Cr-release assay with a xenogeneic murine YAC lymphoma target-cell line. Spleen and non-adherent peritoneal cell suspensions were

prepared from 6 rats per group and erythrocytes in the splenic suspensions were lysed as described previously (Vos *et al.*, 1984). Viable cells were counted in a chamber using the trypan blue exclusion method, and the total number of isolated viable nucleated cells was calculated. YAC cells of an *in vitro* cultured murine cell line of A/Sn Moloney leukemia origin were used as target cells (Kiessling *et al.*, 1975). The effector to target cell (E:T) ratios used were 100, 50 and 25. The methods for labeling target cells with radioactive sodium chromate (^{51}Cr) and details of the cytolytic assay are described elsewhere (de Jong *et al.*, 1980). Specific release was calculated by subtracting the spontaneous release of 6.5% (6.5 ± 1.3 %, n=30) from the experimental release (mean value of duplicate cultures). The specific release was also calculated per whole spleen.

Antibody response to sheep red blood cells (SRBC). At day 28 of the study, rats were injected intraperitoneally either with 2.0 ml of a 50% SRBC suspension, 0.5 ml of a 20%, or 0.5 ml of a 2% suspension in saline (8 rats per group per SRBC dose), followed by an ip booster injection of 50µl of a 20% suspension 15 days later. The SRBC suspension in Alsever's solution was washed three times before the desired suspensions for injection were prepared with saline. The primary antibody response was determined at day 10 after immunization and the secondary response 7 days after the booster injection. Blood was collected from the orbital plexus. Specific serum IgM and IgG titers were determined by ELISA (van Loveren *et al.*, 1991) using the previously specified peroxidase-labeled sheep anti-rat IgG and goat anti-rat IgM. Each serum was titrated starting at a 1:2 dilution, and calculations were performed as described for the anti-*T. spiralis* determinations.

Statistical analysis. Group means were analysed for significance of difference by the two-tailed Student's *t*-test. Data were considered significant when $p<0.05$.

Results

Food and water intake, body weight gain. No significant differences were observed in water intake, food intake and body weight gain of the animals in the control and AC- or THI-exposed groups (data not presented).

White blood cell and blood lymphocyte counts. At day 15 of exposure in the first study, total white blood cell counts in 0.4 and 4% AC-exposed rats were decreased to 85 ($p<0.05$) and 55% ($p<0.001$) of the control counts, respectively. At day 28 of exposure in the second study of the clearance of *L. monocytogenes*, total white blood

cell counts were decreased to 64 ($p<0.001$) and 38% ($p<0.001$) in 0.4 and 4% AC-exposed animals, due to a decrease in lymphocyte counts to 63 ($p<0.001$) and 36% ($p<0.001$) of the controls, respectively.

Yield of muscle larvae and class-specific antibody responses after Trichinella spiralis infection. Results of the study on the resistance to *T. spiralis* infection are presented in Table 3.1. Determination of the yield of muscle larvae 42 days after oral infection of control and AC-exposed rats indicated a dose-related increase in mean larvae count, an effect that was statistically significant in rats exposed to 4% AC. In comparison to the controls, the number of larvae in this group was 34% higher. The anti-*T. spiralis* IgE titers in these animals were significantly decreased both at day 14 and 42 after infection. Anti-*T. spiralis* IgM, IgG and IgA titers did not differ significantly between the groups at either of the days.

Clearance of Listeria monocytogenes. Results of the determination of *L. monocytogenes* counts in spleens of control and AC-exposed rats at day 2 (study 1 and 2) and day 5 (study 2) after infection are shown in Table 3.2. In both studies, an increased capacity of AC-exposed rats to clear *L. monocytogenes* was observed. This

TABLE 3.1

YIELD OF MUSCLE LARVAE AND CLASS-SPECIFIC ANTIBODY RESPONSES IN *TRICHINELLA SPIRALIS* INFECTED CONTROL AND AC-EXPOSED RATS FED A DIET LOW IN VITAMIN B6 [a].

Group	Day after infection	Count of muscle larvae ($x10^3$)	^{2}Log antibody titer			
			IgM	IgG	IgA	IgE
Control	14		3.6 ± 1.3	6.3 ± 1.8	5.3 ± 3.4	2.5 ± 2.4
0.4% AC	14		3.7 ± 1.4	6.2 ± 2.1	5.9 ± 3.8	1.2 ± 0.4
4% AC	14		4.3 ± 1.8	5.4 ± 2.7	6.2 ± 4.2	1.0 ± 0.0 *
Control	42	68 ± 24	4.8 ± 1.7	11.1 ± 1.0	4.1 ± 1.2	6.3 ± 2.2
0.4% AC	42	79 ± 24	6.1 ± 2.2	10.8 ± 1.2	5.1 ± 2.7	5.3 ± 2.4
4% AC	42	91 ± 22 *	5.2 ± 1.4	10.8 ± 0.8	5.3 ± 2.7	3.0 ± 1.5 ***

[a] Weanling rats fed a diet with 2 to 3 ppm pyridoxine were exposed to 0.4 or 4.0% AC in drinking water. At day 28 of exposure, the animals were infected orally with 1000 *T. spiralis* larvae. The data are presented as mean ± SD of 12 rats per group.

* $p<0.05$; *** $p<0.001$, two-tailed Student's *t*-test.

TABLE 3.2

IN VIVO CLEARANCE OF *LISTERIA MONOCYTOGENES* IN CONTROL AND AC-EXPOSED RATS FED A DIET LOW IN VITAMIN B6 [a].

	Listeria count (^{10}Log number/spleen)		
	Study 1	Study 2	
Group	Day 2	Day 2	Day 5
Control	5.75 ± 0.21	5.92 ± 0.25	3.27 ± 1.43
0.4% AC	5.59 ± 0.34	5.51 ± 0.09 ***	1.85 ± 0.15 **
4% AC	5.46 ± 0.39 *	5.65 ± 0.27 *	2.20 ± 0.59

[a] Weanling rats fed a diet with 2 to 3 ppm pyridoxine were exposed to 0.4 or 4.0% AC in drinking water. At day 28 of exposure, the animals were injected iv with $5x10^5$ *L. monocytogenes* bacteria. The data are presented as mean ± SD of 12 (study 1), or 9 to 10 (study 2) rats per group.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$, two-tailed Student's *t*-test.

effect was already noted in the animals exposed to 0.4% AC. At day 8 after infection in study 2, one animal in the 0.4% group had a *Listeria* count of 3.58 (Log/spleen). Apart from that, *Listeria* counts in the 3 groups were all below 10^2 per g spleen at the days 8 and 14 after infection in study 2 (data not presented).

Natural cell-mediated cytotoxicity of spleen and peritoneal cells. The results of the natural cell-mediated cytotoxicity study with spleen cells are presented in Table 3.3. The percentage specific release per culture did not differ between the groups at any of the three effector to target cell (E:T) ratios studied. Because the mean number of viable cells isolated from the spleen demonstrated a dose-related decrease, the difference being statistically significant in the 4% AC group, the Natural Killer activity was also calculated per whole spleen. The mean specific release per spleen demonstrated a dose-related decrease in the AC-exposed rats at all E:T ratios, an effect that was statistically significant in the 4% AC-exposed rats at the E:T ratios of 100 and 50 (Table 3.3). Results of the natural cell-mediated cytotoxicity study with non-adherent peritoneal cells as shown in Table 3.4 demonstrated that the percentage specific release per culture at the various E:T ratios did not differ significantly between the three groups. In addition, the number of cells that was harvested from the peritoneal cavity was not affected significantly.

TABLE 3.3

NATURAL CELL-MEDIATED CYTOTOXICITY OF SPLEEN CELLS FROM CONTROL AND AC-EXPOSED RATS FED A DIET LOW IN VITAMIN B6 [a].

Group	No. of viable cells isolated (x10^7)	E:T ratio 100	50	25
		Specific release (%) per culture		
Control		26.8 ± 6.2	23.4 ± 7.2	21.7 ± 14.9
		25.8 ± 8.7	21.5 ± 7.3	17.7 ± 7.2
		28.3 ± 8.1	22.9 ± 7.7	22.5 ± 7.2
		Specific release per spleen as percentage of mean value of the control group		
		100 ± 49	100 ± 69	100 ± 114
		58 ± 71	46 ± 45	37 ± 31
		30 ± 17 **	25 ± 14 *	27 ± 17

3 ppm pyridoxine were exposed to 0.4 or 4.0% AC in osure, cell suspensions were made of the spleen. Natural ells was measured in a 4-hr ^{51}Cr-release assay with a line. Data are presented as mean ± SD of 6 rats per group. nt's *t*-test.

TABLE 3.4

OTOXICITY OF NON-ADHERENT PERITONEAL CELLS XPOSED RATS FED A DIET LOW IN VITAMIN B6 [a].

		E:T ratio 100	50	25
		Specific release (%) per culture		
Control	83 ± 13	15.8 ± 5.4	7.2 ± 2.4	3.7 ± 1.7
0.4% AC	113 ± 56	12.2 ± 7.5	7.7 ± 5.4	3.9 ± 2.1
4% AC	115 ± 58	10.8 ± 4.9	4.9 ± 2.0	2.5 ± 1.8

[a] Weanling rats fed a diet with 2 to 3 ppm pyridoxine were exposed to 0.4 or 4.0% AC in drinking water. After 28 days of exposure, cells were harvested from the peritoneal cavity. Natural cell-mediated cytotoxicity of the non-adherent peritoneal cells was measured in a 4-hr ^{51}Cr-release assay with a murine YAC lymphoma target-cell line. Data are presented as the mean ± SD of 6 rats per group.

Antibody response to sheep red blood cells (SRBC). The results of the determination of IgM and IgG titers in the primary and secondary immune response to SRBC are given in Table 3.5. The IgM titers in the primary as well as in the secondary response were not affected significantly in THI-exposed rats. However, mean IgG titers in both the primary and secondary response were markedly decreased in rats that had the low doses of SRBC at primary immunization. This effect was statistically significant in the primary response upon immunization with 0.5 ml of a 2% SRBC suspension and in the secondary response in rats that received a dose of 0.5 ml of a 20% SRBC suspension at primary immunization. The IgG titers in the primary and secondary response in animals that received the high dose of SRBC (2 ml of a 50% suspension) at primary immunization were not affected by exposure to THI.

TABLE 3.5

ANTIBODY RESPONSES TO SHEEP RED BLOOD CELLS IN CONTROL AND THI-EXPOSED RATS FED A DIET LOW IN VITAMIN B6 [a].

	Dose of SRBC at primary immunization		$\frac{2}{}$Log antibody titer			
			Primary response		Secondary response	
Group	ml	suspension	IgM	IgG	IgM	IgG
Control	0.5	2%	4.3 ± 1.0	5.5 ± 3.1	2.1 ± 2.6	6.3 ± 4.3
5.72 ppm THI	0.5	2%	4.3 ± 1.2	2.5 ± 1.9 *	1.4 ± 1.7	2.8 ± 2.3
Control	0.5	20%	4.8 ± 2.0	4.5 ± 3.8	2.8 ± 2.4	6.5 ± 3.7
5.72 ppm THI	0.5	20%	4.8 ± 1.7	2.1 ± 1.7	2.4 ± 2.3	1.4 ± 0.5 **
Control	2.0	50%	4.8 ± 1.0	5.8 ± 2.6	2.5 ± 2.3	7.1 ± 3.2
5.72 ppm THI	2.0	50%	5.9 ± 1.6	5.6 ± 1.6	4.3 ± 2.0	6.3 ± 3.1

[a] Weanling rats fed a diet with 2 to 3 ppm pyridoxine were exposed to 5.72 ppm THI in drinking water. At day 28 of exposure, the animals were immunized ip with sheep red blood cells, followed by a booster injection (0.05 ml of a 20% suspension) 15 days later. Blood was collected on days 10 and 22 after primary immunization. Data are presented as mean ± SD of 8 rats per group.

* $p<0.05$; ** $p<0.01$, two-tailed Student's *t*-test.

Discussion

In the present studies, effects of AC and THI on the specific antibody responses to antigens of *T. spiralis* and SRBC have been investigated. The antibody response to these antigens is known to be absent in athymic nude rats (Vos *et al.*, 1980, Terada *et al.*, 1980), indicating that the response is thymus dependent and requires cooperation of T-helper cells. The results of the present study using the *T. spiralis* infection model (Table 3.1) show that mean IgM, IgG and IgA titers to *T. spiralis* were not affected by exposure to AC. However, the IgE response to *T. spiralis* was markedly suppressed at day 14, and in particular at day 42 after infection. In addition to the suppressed IgE response, an increased yield of muscle larvae 42 days after infection of AC-exposed rats indicated a reduced resistance to *T. spiralis* infection. The increased yield of muscle larvae may be due to the suppressed IgE response, since a selective suppression of the IgE antibody response in rats has been reported to result in a diminished resistance and eosinophil response to *T. spiralis* infection (Dessein *et al.*, 1981). Beside humoral immunity, cell-mediated immunity plays a role in the resistance to *T. spiralis* (Larsh *et al.*, 1974). Since AC and THI are known to affect the number of lymphocytes in all subsets determined (Chapter 2), an impaired cell-mediated immunity may have contributed to the diminished resistance to *T. spiralis*. The study on the antibody response to SRBC (Table 3.5) demonstrated that the IgM response is not affected in THI-exposed rats. However, the primary and secondary IgG response to SRBC appeared to be suppressed in rats that received low doses of SRBC at primary immunization. An excess of SRBC at primary immunization was observed to nullify the functional disturbance in THI-exposed rats with respect to the specific immunoglobulin response and resulted in specific IgG titers comparable to those in control animals. The synthesis of the IgG and IgE classes of immunoglobulins is relatively T cell-dependent, since the plasma cellular switch from IgM to IgG and IgE synthesis requires T cell help. The suppressed IgG response to SRBC, and the reduced IgE response to *T. spiralis* in AC- or THI-exposed rats may therefore be caused by a reduced or changed T-helper cell function. Changed T-helper cell function is also suggested by results demonstrating a selective decrease in total IgA levels in AC- and THI-exposed rats (Chapter 2; Houben, unpublished results). The total immunoglobulin levels in rats that have not been infected or immunised reflect the humoral responses upon a natural multiple antigenic stimulation. A reduced T-helper cell function may be caused by a reduction in the number of T-helper cells. Support for this suggestion is obtained from the AC- and

THI-induced reduction in the number of lymphocytes in blood and peripheral lymphoid organs, which was found to be accompanied by a somewhat stronger decrease in the number of CD4+ T (-helper) cells in comparison to the decrease in CD8+ T (-suppressor/cytotoxic) cells (Gobin *et al.*, 1989; Chapter 2). However, a decreased T-helper cell function may also be caused by other mechanisms like, for instance, a change in function or activity of antigen presenting cells or by a disturbed intercellular communication.

Studies using the *L. monocytogenes* infection model (Table 3.2) demonstrated an increased capacity of AC-exposed rats to clear these bacteria, an effect that was noted already in the 0.4% exposed group. The increased clearance was apparent already 2 days after infection. The increased capacity to clear *L. monocytogenes* shortly after infection indicates an increased activity of macrophages towards these bacteria. Interestingly, immunohistochemical studies on the effects of AC and THI in rats demonstrated a remarkable decrease in the number of macrophages positive for the antigen recognized by the monoclonal antibody ED2 in the thymic cortex and in peripheral lymphoid tissues (Chapter 2; Houben, unpublished results). Results from staining with other monoclonal antibodies detecting macrophages indicated that the number of macrophages was not markedly changed, suggesting that only the expression of the antigen recognized by ED2 was reduced (Chapter 2). Since ED2 is considered to recognize a membrane antigen on well differentiated macrophages, as indicated by a higher expression in a steady state population of macrophages in comparison to inflammatory macrophages, it was suggested that the decrease in ED2 expression may be indicative of an altered state of activity or shift in function of the total population of macrophages (Chapter 2). The increased capacity of AC-exposed rats to clear *Listeria* shortly after infection observed in the present studies is supportive for the suggested change in macrophage activity.

Results of the determination of natural cell-mediated cytotoxicity of spleen and non-adherent peritoneal cells (Tables 3.3 and 3.4) demonstrated that AC induced a strong decrease in the Natural Killer (NK) activity in the spleen on a whole-organ basis. The NK activity of spleen cells on a per cell basis and the NK activity of non-adherent peritoneal cells were not affected. The unchanged specific release per culture suggests that the reduced activity per whole spleen is due to a decrease in the number of NK cells. An unaffected NK cell number would implicate that by coincidence, a reduced activity of NK cells is counterbalanced exactly by a relative increase in the number of NK cells due to a reduction in the number of T and B

lymphocytes. However, whether the reduced NK activity in the spleen is caused by a reduction in the number of NK cells or by a reduced activity of the NK cells remains unknown. Data on NK activity after exposure to AC or THI have not been reported before. However, studies on the effects of THI on lymphocyte subsets in peripheral blood of the rat indicated that THI did not affect MARK1$^-$, OX19$^-$ lymphocytes (null cells) and that the null cells became the predominant population after 7 days of exposure (Gobin *et al.*, 1989). In that study, most null cells had large granular lymphocyte characteristics, which made the authors tentatively suggest that NK cells may constitute a proportion of THI-resistant cells. However, no functional assays were carried out in that study. Moreover, since as yet, cells with NK activity in the rat can not be characterised phenotypically, possible quantitative changes in cells with NK activity can not be excluded either. Because effects of AC and THI on the rat immune system are similar (Chapter 2), the results of the present study with AC are in contrast with the suggestion that NK cells are not affected by THI (Gobin *et al.*, 1989).

From the present immune function studies it can be concluded that THI or AC may influence various immune function parameters in rats fed a diet relatively low, but not deficient (Driskell *et al.*, 1973; van den Berg *et al.*, 1982) in vitamin B6. Thymus-dependent immunity was suppressed, whereas parameters of the non-specific resistance were also affected as shown by a decreased NK activity in the spleen and an enhanced clearance of *Listeria*. It is unlikely that the decrease in NK activity is a consequence of the suppressed thymus-dependent immunity, because cellular immunodeficiency is known to be counterbalanced by increased activity of NK cells in the athymic nude rat (de Jong *et al.*, 1980). Several of the effects of AC and THI on rat immune functions may be caused by a change in macrophage function. In particular, the increased capacity of AC-exposed rats to clear *Listeria* that was apparent already within two days after infection, indicates a change in macrophage activity. Previously, effects of AC and THI on lymphoid organs of the rat have been suggested to be caused by a change in activity and differentiation of macrophages, resulting in a disturbance in immunoproliferative processes and in a disturbed regulation of proliferation and maturation of thymocytes (Chapter 2). Moreover, *ex vivo* studies (Chapter 4) recently demonstrated an inability of splenic cells from THI- or AC-exposed rats to respond to mitogenic stimulation, an effect that was due, at least in part, to a suppressive activity of adherent cells as was demonstrated by reconstitution of non adherent cells from control animals with adherent cells from

THI-exposed rats. Currently, research is in progress to study macrophage functions in connection with changed immune functions in AC- and THI-exposed animals.

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Chapter 4

In vitro and *ex vivo* studies with 2-acetyl-4(5)-tetrahydroxybutyl-imidazole (THI), the lymphopenic factor in the colour additive Caramel Colour III

Geert F. Houben[1,2,3], Wouter Lippe[1], Henk van Loveren[3], Willem Seinen[1], and André H. Penninks[1,2]

1: Research Institute of Toxicology, Department of Immunotoxicology, University of Utrecht, Utrecht, The Netherlands
2: TNO Toxicology and Nutrition Institute, Zeist, The Netherlands
3: National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands

Abstract

Caramel Colour III has been observed to induce a reduction in blood lymphocyte counts in rats and in mice. Beside lymphopenia, several other effects on the rat immune system have been observed. The effects are caused by 2-acetyl-4(5)-tetrahydroxybutyl-imidazole (THI), and occur especially in animals fed a diet low in vitamin B6. To obtain more information with respect to mechanisms involved in THI-induced effects on the immune system, several *in vitro* and *ex vivo* studies with splenic cells and thymocytes were conducted. In *in vitro* studies, no effects of THI on cell viability or macromolecular synthesis were observed. However, in *ex vivo* studies with splenic cells from 5.72 or 11.44 ppm THI- or 4% Caramel Colour III-exposed rats (exposure in drinking water) fed a diet low (2-3 ppm) in vitamin B6, an impaired response to mitogenic stimulation with phytohaemagglutinin, concanavalin A or lipopolysaccharide was observed. In *ex vivo* studies with rats fed a diet high (11-12 ppm) in vitamin B6, THI also caused an impaired response to mitogenic stimulation, but the effect was less pronounced. The effect was not caused by changed optimum concentrations of the mitogens or by a change in kinetics of the response to mitogenic stimulation, but was due, at least in part, to a suppressive activity of adherent cells, as was demonstrated by reconstitution of non adherent cells from control animals from an inbred strain using adherent cells from THI-exposed rats. The effect was not prevented by incubation with indomethacin or by culturing the cells in media high in vitamin B6. Thymocytes from THI-exposed rats demonstrated an increased mitogen-induced thymidine-incorporation.

Introduction

Caramel Colours (E150) are among the oldest and most commonly used colour additives in human food stuffs and drinks. Their use accounts for at least 95% by weight of the permitted colour additives used in food. Based on the the reactants used for their production, commercial Caramel Colours have been classified into four classes. Among these, the use of Caramel Colour III, also known as Ammonia Caramel (Colour), accounts for 20 to 25% of the total use of Caramel Colours in the USA and for about 50% in Europe.

Administration of Caramel Colour III has been observed to induce a reduction in blood lymphocyte counts in rats (Evans *et al.*, 1977; Gaunt *et al.*, 1977; Noltes and

Chappel, 1985; Sinkeldam *et al.*, 1988; Chapter 2) and in mice (Iscaro *et al.*, 1988; Chapter 6). Beside lymphopenia, reduced numbers of nucleated cells in peripheral lymphoid organs, changes in thymus morphology and changes in macrophage subpopulations as well as in lymphocyte subset distribution have been observed (Chapter 2). Moreover, immune function tests demonstrated a suppressed thymus-dependent immunity, whereas parameters of the non specific resistance are also affected as shown by a decreased Natural Killer activity in the spleen and an enhanced clearance of *Listeria monocytogenes* (Chapter 3). The effects are caused by the imidazole derivative 2-acetyl-4(5)-tetrahydroxybutyl-imidazole (THI) (Chapter 2; Kröplien *et al.*, 1985; Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988), and occur especially in animals fed a diet low in vitamin B6 (Chapter 2; Gobin and Paine, 1989; Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988). In order to gain more information on the mechanism of action of THI, several *in vitro* and *ex vivo* studies with splenic cells and thymocytes were conducted. In the present paper, the results of these studies are presented and discussed.

Materials and Methods

Materials. Caramel Colour III was manufactured by CPC (Manchester, UK) and was provided by the British Caramel Manufacturers Association (BCMA, London, UK). The specifications of the Caramel Colour III were as follows: Batch: HT 2207; K_{610} (absorbance at 610 nm, pathlength 1 cm, of an aqueous 0.1% w/v solution of Caramel Colour III): 0.18; % solids: 70.6; % N: 4.2; % S: 0.2; % ammoniacal N: 0.01; 4-methylimidazole content: 882 ppm; THI content:143 ppm. The 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)imidazole (THI), Lot MD-1, was synthesized according to Sweeny (Sweeny *et al.*, 1985) and was a gift of the International Technical Caramel Association (ITCA, Washington, DC, USA). [Methyl-^{3}H]-thymidine (47Ci/mmol), [5,6-^{3}H]-uridine (50Ci/mmol) and L-[U-^{14}C]-leucine (348 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, UK.

Animals and maintenance. In most studies, random-bred male Wistar-derived rats (Harlan CPB, Zeist, The Netherlands) were used. In *ex vivo* studies with enriched cell populations, inbred rats (Lewis, Lew/Ola/Hsd; Harlan Olac Ltd., Blackthorn Bicester, Oxon, England) were used. The rats were housed in groups of four in plastic cages on a bedding of chips of wood in an animal room kept at a temperature of 22 ± 2°C with a relative humidity of 45 to 65%, and a 12-hr light/dark cycle. Semi-purified

casein diet (TNO Toxicology and Nutrition Institute, Zeist, The Netherlands) containing 2 to 3 ppm vitamin B6 (supplemented as pyridoxine·HCl, Merck, Darmstadt, FRG) and slightly acidified demineralized water (with HCl to pH 3.0) whether or not supplemented with Caramel Colour III or THI were provided *ad libitum*. To study the influence of dietary vitamin B6, rats were also fed a diet containing 11 to 12 ppm pyridoxine. The diets high or low in vitamin B6 were fed to the animals for at least 14 days prior to the experiments. To investigate whether THI has a direct cytotoxic and/or anti-proliferative effect on lymphoid cells, several *in vitro* studies with cells from thymus and spleen were conducted. In these studies, untreated male Wistar rats weighing 100-170 grams were used. In *ex vivo* studies, groups of 4 rats weighing between 100 and 250 grams were exposed to 4% Caramel Colour III, 5.72 ppm THI or 11.44 ppm THI in drinking water (5.72 ppm THI equals the 4% Caramel Colour III solution) for time periods up to 14 days. Control rats received no Caramel Colour III or THI. Within each study, the variation in body weight of the animals was always below 50 grams. The animals were randomly allocated to treatment and control groups. Cells isolated from thymus glands or spleens of the control and exposed rats were used in mitogenic stimulation assays.

Preparation of cell suspensions. Immediately after decapitation, thymus glands or spleens were isolated aseptically. The thymus glands were carefully trimmed free from adjoining lymph nodes and the organs were sampled in cooled (4°C) Dulbecco's phosphate-buffered saline (pH 7.4) supplemented with 2 mM D-glucose (D(+)-glucose monohydrate, Merck, Darmstadt, FRG) (Dulbecco's PBS/gluc). The organs were minced in fragments with sterile scissors and were gently passed through a nylon sieve (pore diameter 250 μm). To remove cell clumps, the suspensions were passed through a 25-gauge needle and the cells were washed three times. When mitogenic stimulation assays were to be conducted, the cells were finally taken up in RPMI 1640 (Gibco Limited, Paisley, Scotland) supplemented with 2 mM L-glutamine (Gibco Laboratories, Grand Island, NY, USA), 10% heat-inactivated foetal calf serum (Flow Laboratories, Irvine, Scotland), and either with 50 IU penicillin and 50 μg streptomycin/ml (Flow Laboratories, Irvine, Scotland) or with 5 μg gentamicin/ml (Flow Laboratories, Irvine, Scotland) (complete RPMI). To study the influence of the vitamin B6 content of the culture medium, the cells were taken up in RPMI medium to which no vitamin B6 was added (TNO Toxicology and Nutrition Institute, Zeist, The Netherlands). Supplements as described for the complete RPMI were added to obtain B6 low RPMI. Because the vitamin B6 content

of the foetal calf serum was about 40 µg/l, the B6 low RPMI contained about 4 µg vitamin B6/l. To prepare a control medium with a high content of vitamin B6 (B6 high RPMI), pyridoxine·HCl or pyridoxal·HCl (Sigma Chemical Company, St. Louis, USA) was added to the B6 low RPMI (1.0 and 1.5 mg/l, respectively). Total numbers of nucleated cells were determined using a Coulter Counter model ZF (Coulter Electronics Ltd., Harpender, Herts, UK). Cell viability of freshly prepared suspensions was always more than 95%, as routinely judged by trypan blue exclusion.

In vitro studies with THI on cell viability and spontaneous macromolecular synthesis of thymocytes. To study possible *in vitro* effects of THI on cell viability and spontaneous DNA, RNA and protein synthesis, suspensions of $2x10^7$ thymocytes/ml Dulbecco's PBS/gluc were prepared. The cells were pre-incubated in a shaking bath for 30 minutes at 37°C with graded amounts of THI (0, 2.2, 4.3, 6.5, 8.6, and 12.9 µM). After this pre-incubation period, 1 µCi/ml ^{3}H-thymidine (final concentration: 20 nM), 1 µCi/ml ^{3}H-uridine (final concentration 20nM) or 50nCi/ml ^{14}C-leucine (final concentration 145µM) was added. After an additional 30 and 60 minutes incubation period, quadruplicate samples were taken and the cells were harvested onto glass fibre filters using a multiple cell culture harvester (Skatron, Lierby, Norway) and a 5%-solution of trichloroacetic acid. The filters were dried for 16 hrs at 70°C. Finally, the filters were transferred into scintillation vials containing 5 ml of Betacount scintillation fluid (Baker Chemical Co, Phillipsburg, NY, USA) and the amount of label incorporated was counted in a liquid scintillation counter (Kontron MR 300, Zürich, Switzerland). Unlabeled suspensions were used routinely for determination of cell viability (trypan blue exclusion method) at the end of the incubation period.

In vitro studies with THI on phytohaemagglutinin-stimulated DNA synthesis in cells from thymus and spleen. To study a possible *in vitro* effect of THI on the phytohaemagglutinin (PHA)-induced proliferation of cells from thymus and spleen, $5x10^5$ cells were incubated in U-formed polystyrene 96 wells-microtiter plates (Greiner Labortechnik, Greiner B.V., Alphen a/d Rijn, The Netherlands) at 37°C and 5% CO_2 for 40 to 42 hours in 175 µl complete RPMI containing graded amounts of THI (0 nM, 0.5 nM, 0.5 µM, and 0.5 mM) and PHA (Wellcome Diagnostics, Dartford, England; 15 and 25 µg/well) or in the absence of THI and PHA. All incubations were carried out in quadruplicate. After this incubation period, 25µl ^{3}H-thymidine in complete RPMI (final activity 2.5µCi/ml and final concentration 0.5 µM) was added

and the amount of label incorporated was measured as described previously after an additional incubation period of 8 hours.

Ex vivo studies with thymus and spleen cells from Caramel Colour III- or THI-exposed rats. The *ex vivo* effects of Caramel Colour III and THI on the proliferative activity of lymphoid cells were studied in mitogenic stimulation assays using cells from thymus glands and spleens of exposed and control rats. The assays were carried out as described for the *in vitro* studies. Beside PHA (25 and 75 μg/well; in assays with thymocytes also 15, 50 and 100 μg/well), concanavalin A (Con A, Calbiochem Corporation, La Jolla, Calif., USA; 0.2 and 0.5 μg/well; in the assays with thymocytes: 0.25, 0.5, 0.75, 1.00, and 1,50 μg/well) and lipopolysaccharide (LPS, Difco Laboratories, Detroit, Mich., USA; 15 and 25 μg/well) were used as mitogen in several studies. In order to investigate the relationship between the observed *in vivo* and *ex vivo* effects of Caramel Colour III and THI, results from *ex vivo* studies were compared between rats fed diets low and high in vitamin B6. To study the influence of the vitamin B6 content of the culture medium, the assays were also carried out with B6 low RPMI whether or not supplemented with pyridoxal or pyridoxine. Because it may take some time before an increase in extracellular vitamin B6 concentration results in an increase in intracellular pyridoxal 5'-phosphate levels, the assays were also conducted with cells pre-incubated for 24 hrs in culture media with high levels of vitamin B6 before the mitogenic stimulation was started. In order to investigate whether the observed *ex vivo* effects are caused (in part) by altered kinetics of the response to mitogenic stimulation, splenic cells from control and exposed rats were incubated in the presence and absence of mitogens for 16, 40, 64 or 112 hrs before label was added. To investigate the relative contributions of adherent cells (AC) and adherent cell-depleted cells (ACDC) to defects observed in the *ex vivo* studies, splenic cells ($5x10^5$) from THI-exposed and control rats from and inbred strain (Lewis) were incubated in complete RPMI in F-formed polystyrene 96 wells-microtiter plates (Greiner Labortechnik, Greiner B.V., Alphen a/d Rijn, The Netherlands) for 1 hr at 37°C and 5% CO_2, in order to allow AC to adhere to the walls. From part of the wells, the ACDC were removed and the wells were washed gently 3 times with medium. The ACDC were either transferred into clean, unused wells or back into the washed wells containing AC. By means of this reconstitution system, all combinations of AC and ACDC from control and THI-exposed rats as well as the unseparated, total suspensions and the ACDC alone were studied in the mitogenic stimulation assays as described. Prostaglandin-dependence of the THI- or

Caramel Colour III-induced disturbance observed in the *ex vivo* studies was investigated by incubation in the presence of indomethacin (Sigma Chemical Company, ST. Louis, USA; 0.05, 0.5, 5 and 50 μM) as well as in the absence of indomethacin. In the *ex vivo* studies, the dried filters were transferred into sample bags containing 10 ml of Betaplate scintillation fluid (LKB Scintillation Products, FSA Laboratory Supplies, Loughborough, Leics., England) and the amount of label was measured in a flat bed Betaplate liquid scintillation counter (1205 Betaplate; Pharmacia LKB Biotechnology, Uppsala, Sweden).

Statistical analysis. The results were analysed for significance of differences by two-tailed student's *t*-test. Data were considered significant when $p<0.05$.

Results

In vitro studies with THI on cell viability and spontaneous macromolecular synthesis of thymocytes. Five independent *in vitro* experiments were conducted to study possible effects of THI on cell viability and spontaneous macromolecular synthesis of thymocytes using THI concentrations up to 12.9 μM. Cell viability was not affected by THI in any of the *in vitro* experiments. Mean cell viability of the suspensions at the end of the incubation period was always more than 94% (data not presented). Moreover, no significant effect of THI on the short term incorporation of thymidine, uridine or leucine was observed (data not presented). Although it was apparent that in the presence of THI, the precursor incorporation tended to be slightly lower during the first 30 minutes, there was no relationship between the degree of this slightly reduced incorporation and the concentration of THI. The slight difference was compensated for during the second 30 minute incorporation period. In the presence of graded amounts of THI, the amount of label incorporated was about the same after the 60 minute incorporation period when compared to the incorporation in the absence of THI.

In vitro studies with THI on phytohaemagglutinin-stimulated DNA synthesis in cells from thymus and spleen. With cells from thymus glands and spleens, three independent experiments were conducted to study possible *in vitro* effects of THI on the phytohaemagglutinin (PHA)-stimulated DNA synthesis. The results of these studies demonstrated that THI *in vitro* up to concentrations of 0.5 mM does not affect the incorporation of thymidine in thymocytes and splenic cells after stimulation with PHA or in the absence of the mitogen (data not presented).

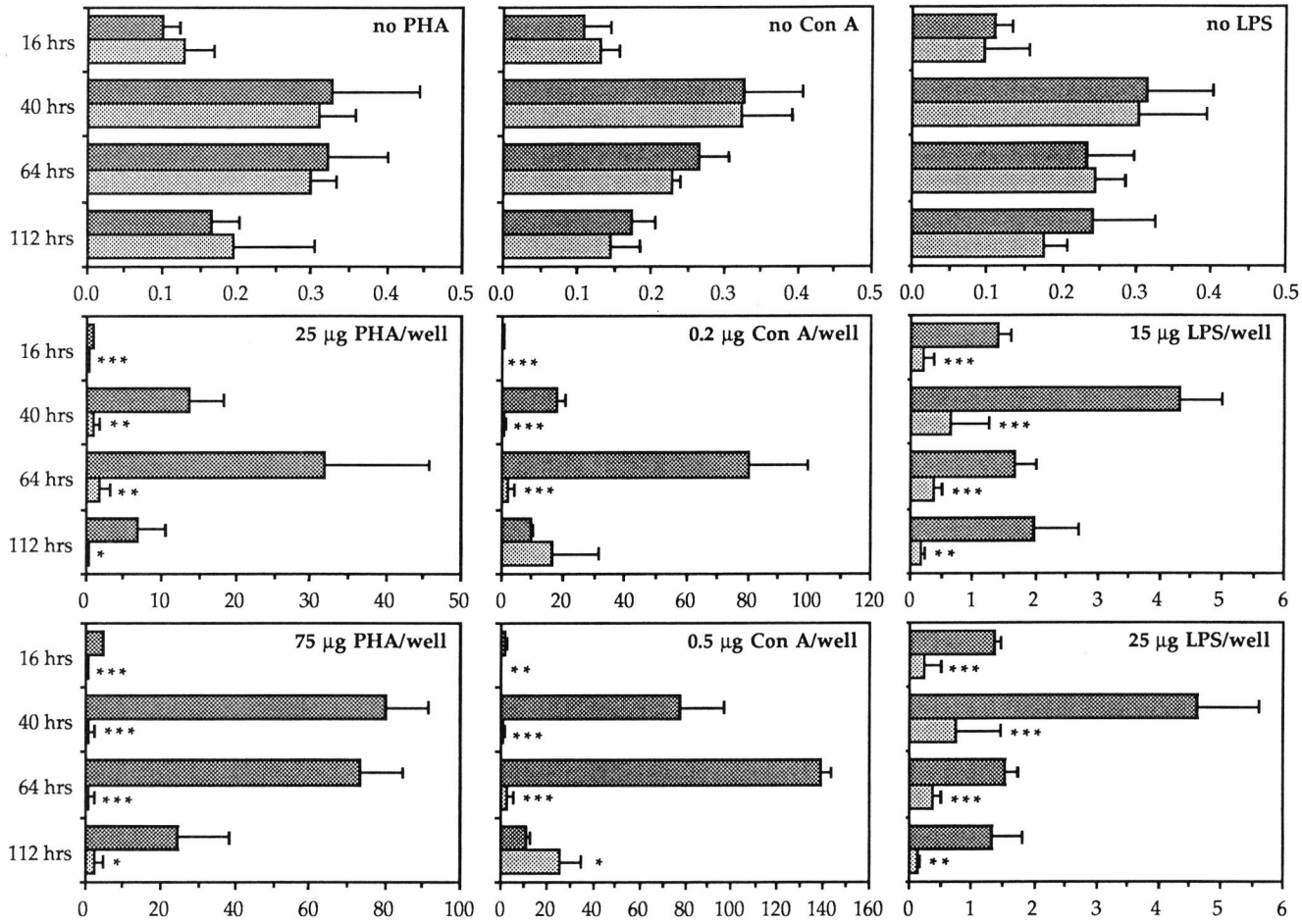

no PHA
no Con A
no LPS
25 µg PHA/well
0.2 µg Con A/well
15 µg LPS/well
75 µg PHA/well
0.5 µg Con A/well
25 µg LPS/well
16 hrs
40 hrs
64 hrs
112 hrs

Figure 4.1

Ex vivo studies with splenic cells. Several *ex vivo* studies on the effects of Caramel Colour III and THI on the mitogen-induced incorporation of thymidine in splenic cells were conducted. In these studies, rats were exposed to 4% Caramel Colour III or to 5.72 or 11.44 ppm THI in drinking water for 3 to 14 days. The results of these studies demonstrated that splenic cells from Caramel Colour III- or THI-exposed rats fed a diet containing 2 to 3 ppm vitamin B6 show a severe impairment in response to mitogenic stimulation with PHA, concanavalin A (Con A) or lipopolysaccharide (LPS), an effect which was already noted after 3 days of exposure. In Figure 4.1, the impaired response to stimulation with PHA, Con A and LPS is illustrated. The Figure presents the results of a study conducted in order to investigate whether the defect may (in part) be caused by altered kinetics of the response to mitogenic stimulation. Splenic cells from control rats and rats exposed to 11.44 ppm THI for 14 days were incubated in the presence and absence of mitogens for 16, 40, 64 or 112 hrs before ^{3}H-thymidine was added. No shift in the kinetics of the response to mitogenic stimulation of splenic cells from THI-exposed rats with PHA or LPS in comparison to the response of control cells was observed. In the case of stimulation with Con A, a prolonged stimulation seemed to occur for splenic cells from THI-exposed rats. However, the relative increase in incorporative activity in comparison to cells from control rats at 112 hours after addition of Con A still resulted in a relatively poor incorporation in comparison to the incorporation by the control cells at 64 hours after addition of the mitogen.

Figure 4.1: Spontaneous and mitogen-induced ^{3}H-thymidine incorporation by splenic cells from control rats (dark bars) and rats exposed to 11.44 ppm THI in drinking water for 14 days (light bars). The animals were fed a diet containing 2 to 3 ppm vitamin B6. Isolated spleen cells were incubated for 16, 40, 64 or 112 hrs in the presence and absence of a mitogen before ^{3}H-thymidine was added, and the amount of label incorporated was determined after an additional incubation period of 8 hrs. All incubations were performed in quadruplicate. The data are presented in 10^{3} cpm/culture ± SD of four rats per group.
PHA: phytohaemagglutinin; Con A: concanavalin A; LPS: lipopolysaccharide.
Differences in incorporation between cells from control rats and cells from rats exposed to THI were analysed by two-tailed student's *t*-test.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

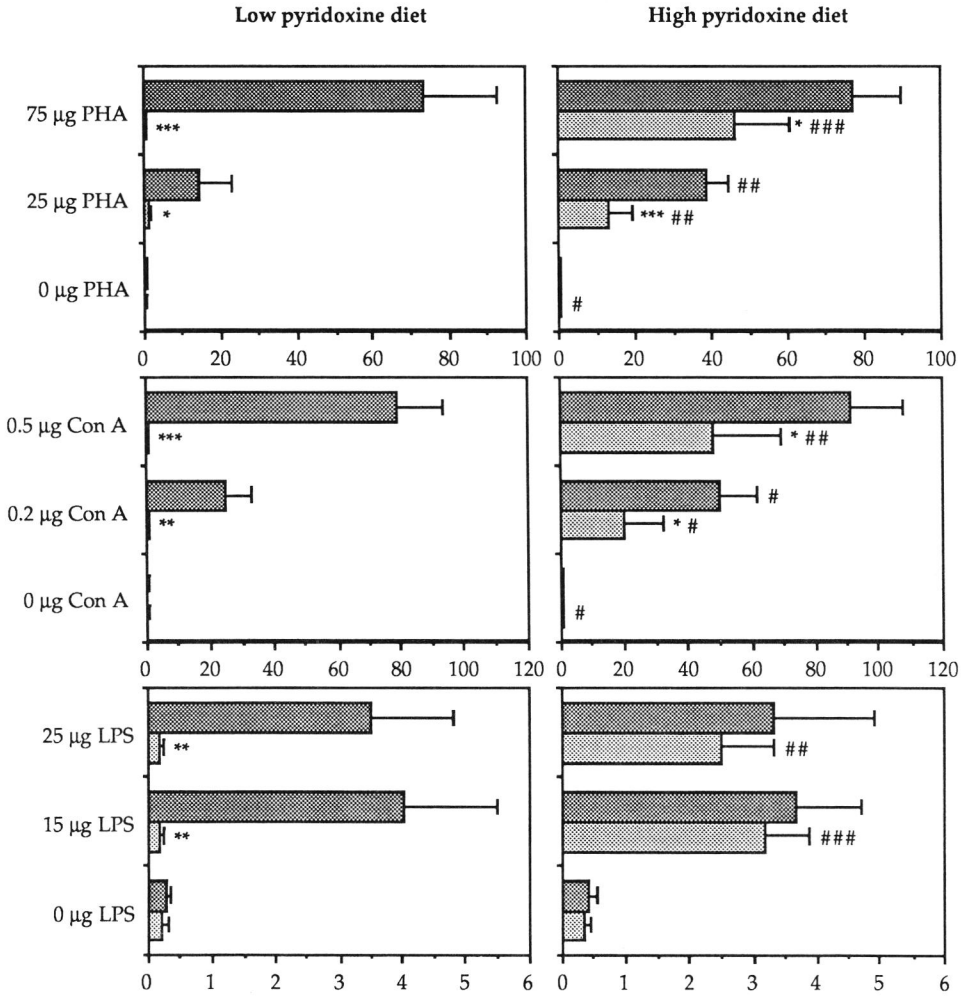


Figure 4.2

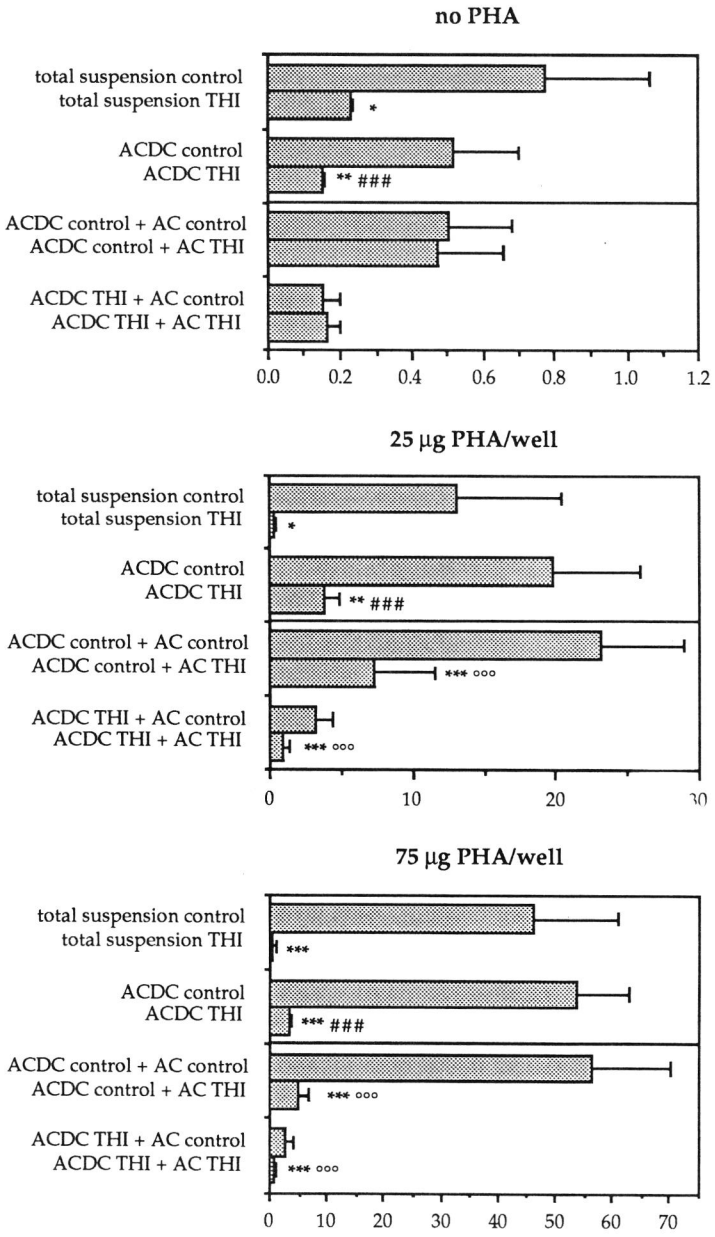
In order to investigate the influence of the dietary vitamin B6 content on the *ex vivo* effects of THI, an *ex vivo* study with rats fed diets low and high in vitamin B6 was conducted. From the results presented in Figure 4.2 it is obvious that splenic cells from THI-exposed rats fed a diet high in vitamin B6 (11-12 ppm pyridoxine) show a better response to mitogenic stimulation in comparison to splenic cells from THI-exposed rats fed a diet low in vitamin B6 (2-3 ppm pyridoxine). However, when

Figure 4.2: Spontaneous and mitogen-induced ^{3}H-thymidine incorporation by splenic cells from control rats (dark bars) and rats exposed to 11.44 ppm THI in drinking water for 14 days (light bars). The animals were fed a diet containing 2 to 3 (low) or 11 to 12 (high) ppm vitamin B6. Isolated spleen cells were incubated for 40 hrs in the presence and absence of a mitogen (concentrations of mitogens indicated in µg/well) before ^{3}H-thymidine was added, and the amount of label incorporated was determined after an additional incubation period of 8 hrs. All incubations were performed in quadruplicate. The data are presented in 10^3 cpm/culture ± SD of four rats per group.
PHA: phytohaemagglutinin; Con A: concanavalin A; LPS: lipopolysaccharide.
Differences in incorporation were analysed by two-tailed student's *t*-test.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$: differences in incorporation between cells from control rats and cells from rats exposed to THI.
# $p<0.05$, ## $p<0.01$, ### $p<0.001$: differences in incorporation associated with the variation in dietary vitamin B6 content.

fed the diet high in vitamin B6, an impaired thymidine incorporation by splenic cells from exposed rats could still be noted upon stimulation with PHA or Con A in comparison to the incorporation by splenic cells from control animals.

To investigate a possible influence of the vitamin B6 content of the culture medium, several studies with culture media variable in vitamin B6 content were conducted. From these studies (data not presented) it was apparent that variations in the pyridoxine or pyridoxal content of the medium did not modulate the *ex vivo* effect of Caramel Colour III and THI. Moreover, pre-incubation of the cells from exposed rats for 24 hrs in 1 mg pyridoxine/l or in 1.5 mg pyridoxal/l medium before the mitogenic stimulation was started did not restore the defect in the response to mitogenic stimulation.

The relative contributions of adherent cell (AC) and adherent cell-depleted cells (ACDC) to the defect observed in the *ex vivo* studies with Caramel Colour III and THI was investigated using combinations of enriched splenic cell populations from control and THI-exposed rats from an inbred strain. The results are presented in Figure 4.3. ACDC from THI-exposed rats incorporated about 19 times more thymidine when compared to the total splenic suspensions from these rats in the


no PHA
total suspension control
total suspension THI
ACDC control
ACDC THI
ACDC control + AC control
ACDC control + AC THI
ACDC THI + AC control
ACDC THI + AC THI
*
** ###
0.0
0.2
0.4
0.6
0.8
1.0
1.2
25 µg PHA/well
total suspension control
total suspension THI
ACDC control
ACDC THI
ACDC control + AC control
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ACDC THI + AC control
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10
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75 µg PHA/well
total suspension control
total suspension THI
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Figure 4.3

Figure 4.3: Spontaneous and phytohaemagglutinin (PHA)-induced ^{3}H-thymidine incorporation by splenic cells from control Lewis rats and Lewis rats exposed to 11.44 ppm THI in drinking water for 14 days. The animals were fed a diet containing 2 to 3 ppm vitamin B6. Isolated spleen cells were incubated for 1 hr at 37°C to allow adherent cells (AC) to adhere in the wells and adherent cell-depleted cells (ACDC) were isolated and incubated alone, or were added to washed AC-containing wells. Total splenic suspensions and ACDC-suspensions, as well as combinations of ACDC and AC were incubated in the presence and absence of PHA for 40 hrs before ^{3}H-thymidine was added, and the amount of label incorporated was determined after an additional incubation period of 8 hrs. Per concentration of PHA, the upper set of bars represents the incorporation by total splenic suspensions and ACDC from control and THI-exposed rats, and the lower set represents the incorporation by ACDC from control and THI-exposed rats after reconstitution with AC from control or THI-exposed rats. All incubations were performed in quadruplicate. The data are presented in 10^{3} cpm/culture ± SD of four rats per group (total and ACDC alone) or of 16 combinations between ACDC and AC. Differences in incorporation were analysed by two-tailed student's *t*-test.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$: effect of THI-exposure on the incorporation by total splenic suspensions and ACDC (upper sets), or difference in impact of reconstitution with AC from control rats and AC from THI-exposed rats (lower sets). ### $p<0.001$: effect of withdrawal of AC from splenic suspension of THI-exposed rats. °°° $p<0.001$: effect of reconstitution of ACDC with AC from THI-exposed rats. Withdrawal of AC from splenic suspensions from control rats, and reconstitution of ACDC from control or THI-exposed rats with AC from control rats did not cause any significant changes.

presence of 25 µg PHA/well, and 7 times more in the presence of 75 µg PHA/well. Reconstitution of these ACDC-suspensions with the AC from the THI-exposed rats resulted in a strong decrease again. Reconstitution of ACDC from control rats with AC from THI-exposed rats resulted in a very strong decrease in the incorporation by the ACDC from the control rats to 37% in the presence of 25 µg PHA/well and to 9% in the presence of 75 µg PHA/well. These results indicate a suppressive activity of

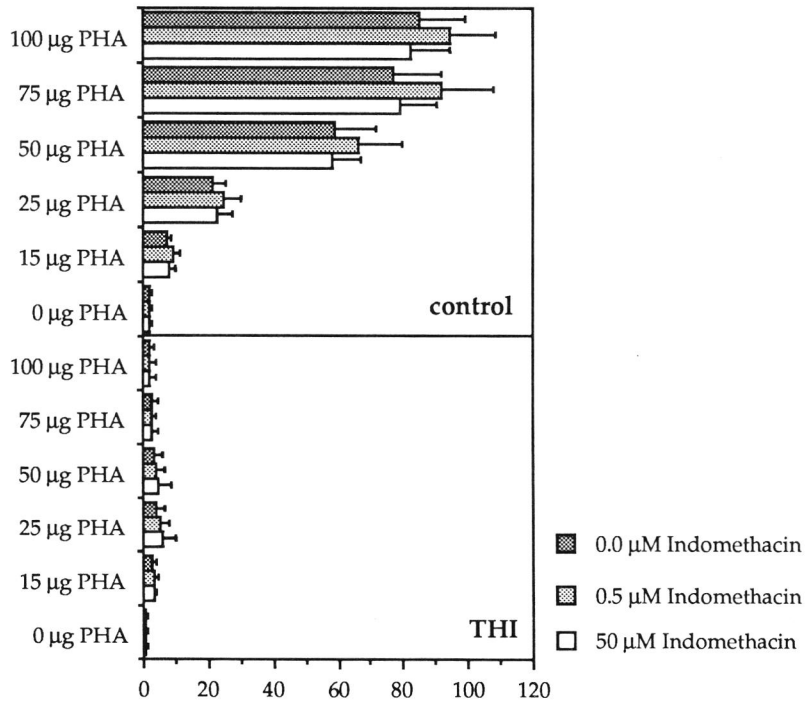

Figure 4.4: Spontaneous and phytohaemagglutinin (PHA)-induced ^{3}H-thymidine incorporation by splenic cells from control rats (upper set) and rats exposed to 11.44 ppm THI in drinking water for 14 days (lower set). The animals were fed a diet containing 2 to 3 ppm vitamin B6. Isolated spleen cells were incubated for 40 hrs in the presence and absence of PHA (concentrations of mitogen indicated in µg/well) and in the presence of 0.5 (grey bars) or 50 (white bars) µM indomethacin or in the absence of indomethacin (dark bars) before ^{3}H-thymidine was added. The amount of label incorporated was determined after an additional incubation period of 8 hrs. All incubations were performed in quadruplicate. Data are presented in 10^{3} cpm/culture ± SD of four rats per group. Differences in incorporation in the presence and absence of indomethacin were analysed by two-tailed student's *t*-test.

AC from THI-exposed animals. Withdrawal of AC from suspensions from control rats, or reconstitution of ACDC from control or exposed rats with AC from control rats did not cause any significant changes.

To investigate the possibility of a prostaglandin-mediated pathway as a cause of the THI- or Caramel Colour III-induced disturbance observed in the *ex vivo* studies, three *ex vivo* studies were conducted in which indomethacin was added to part of the cultures (0.05, 0.5, 5 and 50 μM). The results obtained in the presence of 0.5 and 50 μM indomethacin as well as in the absence of indomethacin from a representative experiment are presented in Figure 4.4. Addition of indomethacin did not restore the defect caused by THI in any of the three studies.

Ex vivo studies with thymocytes. In order to investigate whether *in vivo* exposure to THI has an effect on the response to mitogenic stimulation of thymocytes, two *ex vivo* studies were conducted. In Figure 4.5, the results of one representative study with thymocytes are presented. The results demonstrate a much stronger response to stimulation with PHA or Con A by thymocytes from THI-exposed rats in comparison to thymic cells from control rats.

Discussion

The *ex vivo* studies with Caramel Colour III and THI presented in this paper demonstrated a severe impairment in response to mitogenic stimulation of splenic cells from THI- and Caramel Colour III-exposed rats fed a diet low, but not deficient (Driskell *et al.*, 1973, Van Den Berg *et al.*, 1982) in vitamin B6 (see Figure 4.1 for effect of THI). This defect was not dependent on the mitogen used, and therefore on the type of lymphoid cells stimulated, as was indicated by an equivalent disturbance observed upon stimulation with with phytohaemagglutinin (PHA), concanavalin A (Con A) or lipopolysaccharide (LPS) (Figures 4.1 and 4.2). A similar effect was not observed upon *in vitro* exposure of cells to THI. Moreover, in studies with a combined *in vivo* and *in vitro* exposure to THI (data not presented), no additional effect due to *in vitro* exposure to THI was observed. Results of several additional studies indicated that once the effect has been induced *in vivo*, it is not possible to modulate the defect *in vitro* by changing the pyridoxine or pyridoxal content of the culture medium, even when the cells are pre-incubated in medium with a high content of vitamin B6 for 24 hrs. Because spleen cells from mice fed a diet containing 1.2 ppm vitamin B6 have been reported to give a higher response after stimulation

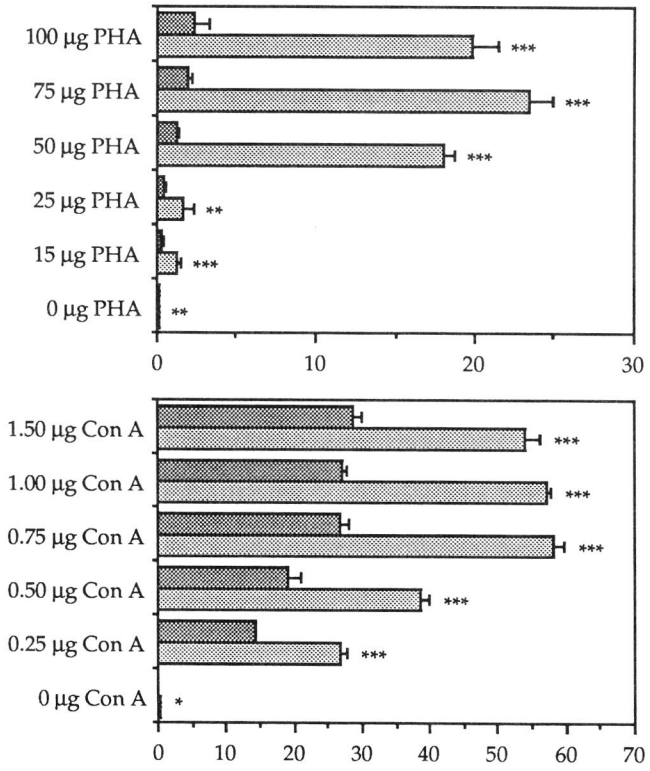

Figure 4.5: Spontaneous and mitogen-induced ^{3}H-thymidine incorporation by thymocytes from control rats (dark bars) and rats exposed to 11.44 ppm THI in drinking water for 14 days (light bars). The animals were fed a diet containing 2 to 3 ppm vitamin B6. Isolated thymocytes were incubated for 40 hrs in the presence and absence of phytohaemagglutinin (PHA) or concanavalin A (Con A) (concentrations of mitogens indicated in µg/well) before ^{3}H-thymidine was added, and the amount of label incorporated was determined after an additional incubation period of 8 hrs. All incubations were performed in quadruplicate. The data are presented in 10^{3} cpm/culture ± SD of four rats per group. Differences in incorporation between cells from control rats and cells from rats exposed to THI were analysed by two-tailed student's *t*-test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

with PHA when cultured in medium with a high content of pyridoxal in comparison to cells cultured in low pyridoxal medium (Gridley *et al.*, 1988), pyridoxal was considered an appropriate supplement of vitamin B6 in these *in vitro* systems. It is well known that dietary pyridoxine to a certain extent is able to prevent the Caramel Colour III- and THI-induced effects in *in vivo* studies (Chapter 2; Gobin and Paine, 1989; Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988). Therefore, the defect in the response to mitogenic stimulation observed in the *ex vivo* studies was studied in an experiment with rats fed diets with varying levels of vitamin B6. The results (Figure 4.2) demonstrated that dietary vitamin B6 only partially prevents the defect induced by THI. This observation is in accordance with results from *in vivo* studies with Caramel Colour III and THI in which pyridoxine only partially prevents effects on the immune system (Chapter 2).

The defect in response to mitogenic stimulation could not be explained by a change in the kinetics of the response to the mitogenic stimulation (Figure 4.1). Although a prolonged stimulation seemed to occur upon Con A-stimulation of cells from exposed rats, the slight relative increase in incorporative activity in comparison to cells from control rats at 112 hours after addition of Con A still resulted in a relatively poor incorporation in comparison to the incorporation by the control cells at 64 hours after addition of the mitogen and may in fact be due to acidification and depletion of the medium of the control cells.

In order to investigate the relative contributions of adherent cells (AC) and adherent cell-depleted cells (ACDC) to the defect observed in the *ex vivo* studies with THI and Caramel Colour III, an *ex vivo* study with rats from an inbred strain (Lewis) was conducted. From recent studies it appeared that effects of THI in Lewis rats (Dr. M.A. Bloksma, University of Utrecht; unpublished results) are similar to those reported in Wistar rats (Chapter 2). From the results of the *ex vivo* studies with Lewis rats (Figure 4.3), it was apparent that AC from THI-exposed rats cause a strong suppression of the proliferative response of lymphoid cells from control animals, indicating that the defect observed in the *ex vivo* studies is caused, at least in part, by a suppressive activity of AC from the THI-exposed rats. This suppression may be due to a changed activity of the AC on a per cell basis. Addition of indomethacin to the cultures (Figure 4.4) did not modulate the *ex vivo* effect, indicating that an excess of prostaglandin production as a major cause of the suppression induced by the AC is not likely. The suppressive activity of the AC may also be a result of a change in subpopulation distribution of splenic cells from THI-exposed rats. THI is known to

induce a decrease in the number of lymphocytes in peripheral lymphoid organs (Chapter 2). As a consequence, wells to which splenic cells from THI-exposed rats were added were expected to contain a larger number of adherent cells. This was confirmed in studies in which the ACDC were counted to determine the percentage of not adhering cells. Whereas 63.5% (± 9.6%) of the nucleated splenic cells from control rats did not adhere, this percentage was decreased to 42.7% (± 3.1%) in rats exposed to 11.44 ppm THI for 14 days ($p<0.05$). Moreover, in *in vivo* studies with rats, Caramel Colour III and THI have been observed to cause a strong decrease in the number of macrophages positive for the monoclonal antibody ED2 in spleen and other lymphoid tissues (Chapter 2). Recently, immunocytochemical characterisation of splenic cells that adhered to glass demonstrated a dramatic decrease in the percentage of $ED2^+$ adhering cells in THI-exposed (11.44 ppm in drinking water for 14 days) rats, from 55.7 ± 9.8% for the controls to 0.3 ± 0.6% for the THI-exposed animals ($p<0.001$). As is obvious from Figure 4.3 also, ACDC from THI-exposed rats still incorporated considerably less thymidine than ACDC from control animals, while AC from control rats were not able to restore the incorporative activity of ACDC from THI-exposed animals. Although it can not be excluded that the lymphoid cells from the THI-exposed rats suffered functional disturbances as well, the difference in incorporation between ACDC from control rats and THI-exposed rats is likely to be due, at least in part, to an incomplete removal of cells with suppressive activity from the spleen cell suspensions from the THI-exposed rats. Immunocytochemical characterisation of ACDC from spleens of control and THI-exposed Wistar rats on cytospin centrifuge preparations using the monoclonal antibodies ED1 and ED2 (Dijkstra *et al.*, 1985) indicated that a considerable percentage of the ACDC was $ED1^+$ and thus belonged to the monocyte-macrophage lineage, while this percentage was increased two fold ($p<0.05$) in ACDC-suspensions from THI-exposed rats in comparison to the controls (Dr. H.J. Bos, University of Utrecht, unpublished data). In those studies, AC were removed according to the same method as described in the material and method section. The percentage of $ED2^+$ cells in the ACDC-suspensions was decreased from 4.3 ± 2.6% for the controls to 0.0 ± 0.0% ($p<0.05$) for THI-exposed rats.

Since the monoclonal antibody ED2 is considered to recognize a membrane antigen on well differentiated macrophages, as indicated by a higher expression in a steady state population of macrophages in comparison to inflammatory macrophages, we suggested previously that the decrease in expression of the antigen

recognised by ED2 may be indicative of an altered state of activity or shift in function of the total population of macrophages (Chapter 2). This suggestion was supported by an observed increased capacity of Caramel Colour III-exposed rats to clear *Listeria monocytogenes* (Chapter 3). Although the expression of the membrane antigen recognised by the monoclonal antibody ED2 has not yet been clearly associated with functional characteristics of the cells, we suggested that the effects of Caramel Colour III and THI on the rat immune system are mediated by the suggested change in macrophage activity, resulting in a change in immunoproliferative processes and in a disturbed regulation of proliferation and maturation of thymocytes (Chapter 2), as well as in changed immune functions (Chapter 3). The present data from the *ex vivo* studies also indicate that (part of) the effects of THI on the rat immune system are mediated by a change in activity of accessory cells.

The causes for the inability of THI to induce an *in vitro* effect may be manifold. One might suggest the need for metabolic conversion of THI. Pilot studies (Houben, unpublished results) using a High-Performance-Liquid-Chromatography (HPLC) separation method with UV-absorbance detection of THI in which rats received a single i.v. injection of THI indicated that THI is cleared from the blood very rapidly. All rats exhibited a rapid decrease of their plasma THI levels with about 50% per 15 to 30 minutes, and analysis of urine samples indicated that within 6 to 8 hours, 65 to 80% of the injected dose of THI could be recovered from the urine. No indications for the existence of metabolites of THI were found. These observations are in accordance with data reported by Phillips and Paine (1990). Therefore, it seems justified to propose that THI itself causes the *in vivo* and *ex vivo* effects. There is evidence (Chapter 2; Chapter 5) suggesting that THI exposure interferes with pyridoxal 5'-phosphate (PLP)-dependent processes. It is possible that THI-induced disturbances in PLP-dependent processes affect in particular one or a few types of cells in specific stages of differentiation, maturation or cellular communication, or that only one or a few PLP-dependent processes are in particular sensitive to THI. Possibly, the *in vitro* assays used do not depend on these cells in these sensitive stages of differentiation, maturation or cellular communication or on these PLP-dependent processes, respectively, which may account for the inability of THI to influence the lymphocyte proliferation in the *in vitro* studies and the inability of vitamin B6 added to the medium to influence the proliferative activity of lymphocytes in *ex vivo* studies.

The results from the *ex vivo* studies with thymocytes, as presented in Figure 4.5, demonstrated a strong increase in thymidine incorporation in cells from THI-

exposed rats. *In vivo* studies with Caramel Colour III and THI (Chapter 2) demonstrated a remarkable decrease in the thymic cortex over medulla area ratio in treated rats, while an increased cell density was observed in the medulla. By means of immunohistochemical staining, it was demonstrated that the increase in medullary cell density was due to an increase in the number of mature medullary thymocytes (Chapter 2). The observed increase in incorporative activity in response to mitogenic stimulation in the *ex vivo* studies with thymocytes confirmed the observations from the immunohistochemical studies and indicates that there is a strong increase in the number of mature thymocytes. Since a reduction in the number of recent thymic emigrants has been demonstrated in peripheral lymphoid tissues of Caramel Colour III- and THI-exposed rats (Chapter 2), the increase in the number of mature thymocytes may (in part) be caused by a diminished migration of mature cells from the thymus to the periphery.

Acknowledgments

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Chapter 5

Immunotoxic effects of 2-acetyl-4(5)-tetrahydroxybutyl-imidazole (THI), and 4'-deoxypyridoxine (DOP) in rats

Geert F. Houben[1,2,3], Monique van Laerhoven[1], Bert W. Lam[1], Henk van Loveren[3], Willem Seinen[1], and André H. Penninks[1,2]

1: Research Institute of Toxicology, Department of Immunotoxicology, University of Utrecht, Utrecht, The Netherlands
2: TNO Toxicology and Nutrition Institute, Zeist, The Netherlands
3: National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands

Summary

Administration of the colour additive Caramel Colour III has been associated with immunotoxicity in rats. The effects are caused by 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI), and occur especially in animals fed a diet with a relatively low content of vitamin B6. The present studies demonstrate that effects of THI on the rat immune system are similar to those of the vitamin B6 antagonist 4'-deoxypyridoxine (DOP). Based on the similarity in effects of THI and DOP on the rat immune system and the protective potency of vitamin B6 on the effects, it is suggested that THI and DOP have the same mechanism of immunotoxicity, accomplished by inhibition of vitamin B6-dependent processes. Because THI does not affect vitamin B6 metabolism and effects of DOP on the rat immune system may not necessarily depend on a decrease in pyridoxal 5'-phosphate (PLP) levels, it is proposed that the THI- and DOP-induced inhibition of vitamin B6-dependent processes is mainly due to a competition between THI or DOP 5'-phosphate and the coenzyme PLP for binding to the cofactor binding-site of one or more PLP-dependent apoenzymes.

Introduction

Administration of the colour additive Ammonia Caramel Colour (Caramel Colour III) has been associated with decreased numbers of blood lymphocytes in rats (Evans *et al.*, 1977; Gaunt *et al.*, 1977; Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988; Chapter 2). Beside lymphopenia, reduced numbers of nucleated cells in peripheral lymphoid organs, changes in thymus morphology and changes in macrophage subpopulations as well as in lymphocyte subset distribution were observed (Chapter 2). Moreover, changes in immune function parameters were demonstrated, among which a severe impairment in the response to mitogenic stimulation of splenic cells from exposed rats (Chapters 3 and 4). The effects are caused by the imidazole-derivative 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI) (Kröplien *et al.*, 1985; Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988; Chapter 2), and occur especially in animals fed a diet with a relatively low content of vitamin B6 (Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988; Gobin and Paine, 1989; Chapter 2).

Because of the interference of vitamin B6 with the lymphopenic potency of Caramel Colour III, the effects of Caramel Colour III and THI on blood lymphocyte

counts have been compared with the effect of the lymphopenic vitamin B6 antagonist 4'-deoxypyridoxine (DOP). Comparison of the lymphopenic potency of Caramel Colour III with that of DOP indicated that a Caramel Colour III containing about 200 ppm THI had a lymphopenic potency comparable to that of a material containing approximately 1000 ppm DOP (Sinkeldam *et al.*, 1988). The effects of DOP, Caramel Colour III and THI on the number of blood lymphocytes could be observed already within 3 days, and were about maximum after 7 days of exposure (Sinkeldam *et al.*, 1988). A more detailed comparison of effects of Caramel Colour III or THI and DOP has not been made so far. Previously, it was suggested that THI disturbs a pyridoxal 5'-phosphate-dependent process (Chapter 2). To gain more information about the mechanism of action of THI, it was considered essential to compare effects of Caramel Colour III or THI and DOP in more detail. In the present paper, results of several studies on DOP and THI are presented and compared.

Material and Methods

Materials. The 4'-deoxypyridoxine (DOP) used in these studies was obtained from Sigma Chemical Company (St. Louis, USA). The 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI), Lot MD-1, was synthesized according to the method described by Sweeny *et al.* (1985) and was kindly provided by the International Technical Caramel Association (ITCA, Washington, DC, USA).

Studies on time-effect relationship with THI. Effects of THI on total and differential leukocyte counts and on thymus and spleen morphology were studied over time using young random-bred male Wistar-derived rats weighing 70 to 80 g (Harlan CPB, Zeist, The Netherlands). The rats were housed in groups of four in plastic cages on a bedding of chips of wood in an animal room kept at a temperature of 22 ± 2°C with a relative humidity of 45 to 65%, and a 12-hr light/dark cycle. Semi-purified casein diet (TNO Toxicology and Nutrition Institute, Zeist, The Netherlands) containing 2 to 3 ppm vitamin B6 (supplemented as pyridoxine·HCl, Merck, Darmstadt, FRG) and slightly acidified demineralized water (with HCl to pH 3.0) whether or not supplemented with 5.72 ppm THI were provided *ad libitum*.

In a first, short-term study, blood samples were collected in heparinised tubes from the tip of the tail of 7 or 8 rats per group after 12, 20, 36, 44 and 84 hrs of exposure and blood smears were made. Blood samples were never collected from the same animals at two subsequent time points. Samples of the decoagulated blood

were used to determine total white blood cell numbers using a Coulter Counter model ZF (Coulter Electronics Ltd., Harpender, Herts, UK). An estimation of the relative numbers of the different types of leukocytes was obtained by counting 200 cells in the blood smears after Pappenheim staining (Gorter and de Graaf, 1955). From the total and the differential leukocyte counts, the absolute numbers of the different types of leukocytes were calculated.

In a second study, with weanling rats weighing 50 to 60 g, blood samples were collected from 9 animals per group for total and differential leukocyte counts after 2, 4, 7, 11, 15, 23, and 29 days of exposure. After each collection of blood, 3 animals per group were killed under ether anaesthesia by exsanguination from the abdominal aorta. Thymi and spleens were isolated and processed for (immuno)histochemical examination. The methods and the panel of mouse anti-rat mAb's used for immunohistochemical staining were as described previously (Chapter 2). In animals exposed to THI for 29 days, recovery from the effects was studied at days 3, 6, and 14 after withdrawal of THI-exposure.

Two-week study on 4'-deoxypyridoxine (DOP). Effects of DOP on the immune system were studied using 6 weeks old random-bred male Wistar-derived rats weighing 125 to 150 g (Winkelmann, Borchen, FRG). The animals were housed in groups of five in wire-mesh floored cages in an animal room maintained at the same conditions as those used for the study on the time-effect relationship with THI. Food containing 2 to 3 ppm vitamin B6 and acidified demineralized drinking water whether or not supplemented with 40 ppm DOP were freely available. Prior to the experimental period, the animals were allocated to control and treatment group by computer randomization and proportionately to their initial leukocyte counts. Individual body weights and food intake on a cage basis were recorded weekly, and liquid intake on a cage basis was determined every day. After 15 days of exposure, blood samples were collected from the tip of the tail of 10 animals per group for determination of total and differential leukocyte counts as described above. Subsequently, the animals were killed under ether anaesthesia by exsanguination from the abdominal aorta. At necropsy, weights of adrenals, kidneys, liver, thymus, spleen and popliteal lymph nodes were recorded. In addition, mesenteric lymph nodes were isolated. Parts of thymus, spleen and lymph nodes from 5 animals per group were fixed in 4% phosphate-buffered formaldehyde for histopathology as described previously (Chapter 2). In addition, cell suspensions were prepared from thymus, spleen, popliteal lymph nodes and bone marrow of 5 animals per group as

described (Chapter 2). The total numbers of nucleated cells isolated from these tissues was determined using the Coulter Counter.

Study on time-effect relationship with DOP. Effects of DOP on thymus and spleen were studied over time using young random-bred male Wistar-derived rats weighing 80 to 100 g (Harlan CPB, Zeist, The Netherlands). Part of the animals was exposed to 40 ppm DOP in drinking water for 2, 4, 7, 14 or 28 days. The maintenance of the animals was as described for the study on the time-effect relationship with THI. At the respective days, a blood sample was taken from the tip of the tail of 3 exposed and 2 control animals for total leukocyte determination using the Coulter Counter. Subsequently, the animals were killed and thymi and spleens were isolated and processed for immunohistological examination using the monoclonal antibodies as described previously (Chapter 2).

Ex vivo and in vitro studies on DOP. The response of splenic cells and thymocytes from control and DOP-exposed rats (40 ppm in drinking water for 14 days) on mitogenic stimulation with phytohaemagglutinin (PHA), concanavalin A (Con A) and lipopolysaccharide (LPS; only in the assays with splenic cells) were studied using random-bred male Wistar-derived rats weighing 100 to 150 g (Harlan CPB, Zeist, The Netherlands) fed a diet containing 2 to 3 ppm vitamin B6. In addition, a possible *in vitro* effect of DOP on the response of splenic cells to stimulation with PHA was studied by incubation in the presence of DOP at concentrations up to 1.0 mM. The maintenance of the animals, the experimental protocols and the materials and methods used were as described previously (Chapter 4).

Statistical analyses. Group means were analysed for significance of difference by the two-tailed Student's *t*-test. Data were considered significant when $p<0.05$.

Results

Studies on time-effect relationship with THI. Results on total leukocyte, blood lymphocyte and neutrophil counts in the short-term study are presented in Table 5.1. Within 20 hrs, a significant decrease in blood lymphocyte counts was observed in THI-exposed rats when compared to lymphocyte counts in control animals. The absolute number of neutrophils was increased significantly within 12 hrs. Total leukocyte numbers in THI-exposed rats did not differ significantly from those in control animals throughout the study. No significant differences were observed in blood eosinophil, basophil or monocyte numbers (data not presented).

TABLE 5.1

TOTAL LEUKOCYTE, BLOOD LYMPHOCYTE AND NEUTROPHIL NUMBERS OF CONTROL AND SHORT-TERM THI-EXPOSED RATS FED A DIET LOW IN VITAMIN B6 [a].

Parameter	Group	Time point				
		12 hrs	20 hrs	36 hrs	44 hrs	84 hrs
leukocytes	control	24.1 ± 4.9	20.5 ± 3.5	23.0 ± 4.2	18.1 ± 6.6	21.4 ± 5.5
	THI	22.5 ± 3.8	21.2 ± 3.4	23.9 ± 3.5	15.1 ± 2.8	17.6 ± 5.3
lymphocytes	control	21.3 ± 4.4	18.7 ± 3.2	20.9 ± 4.0	16.4 ± 6.2	18.2 ± 3.3
	THI	18.4 ± 3.1	15.2 ± 2.3 *	15.5 ± 2.8 **	8.3 ± 1.5 **	9.8 ± 2.1 ***
neutroplils	control	2.3 ± 1.1	1.4 ± 0.5	1.8 ± 0.5	1.2 ± 0.2	1.9 ± 0.5
	THI	3.5 ± 1.1 *	5.6 ± 1.6 ***	7.8 ± 1.3 ***	6.3 ± 1.5 ***	5.1 ± 1.8 ***

[a] Rats fed a diet low (2-3 ppm) in pyridoxine were exposed to 5.72 ppm THI in drinking water. The data are presented as mean ± SD of 7 or 8 rats per group and are expressed in 10^6 cells/ml.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$, two-tailed Student's *t*-test.

In the 29-day study, the results on total and differential leukocyte counts (data not presented) were, in general, in line with those from the short-term study. Blood lymphocyte numbers in exposed animals were decreased significantly already at day 2 (73% from control numbers; $p<0.05$), and persisted at this level until withdrawal of THI-exposure. Although the number of neutrophils tended to be increased at day 2 (135% when compared to control numbers; $p>0.05$), the level of statistical significance was reached at day 4 (236%; $p<0.001$). The number of neutrophils remained increased, fluctuating between 162 and 333% when compared to the control numbers until withdrawal of THI-exposure. Total leukocyte numbers and blood eosinophil, basophil and monocyte numbers did not differ significantly between exposed and control animals throughout the study.

In thymus sections of THI-exposed rats, a decrease in cortical area was observed. The medullary area was increased and was more densely packed with cells. By means of immunohistochemistry, the increase in medullary cell density was found to be mainly due to an increase in $CD4^+$ ($ER2^+$; reference: Joling *et al.*, 1985), $OX44^+$ (Paterson *et al.*, 1987; Paterson and Williams, 1987) and $HIS45^+$ (Kampinga *et al.*, 1990) thymocytes. In the cortex of the thymus, a strong decrease in the number of

TABLE 5.2

TERMINAL BODY WEIGHTS AND ABSOLUTE AND RELATIVE ORGAN WEIGHTS OF CONTROL AND DOP-EXPOSED RATS FED A DIET LOW IN VITAMIN B6 [a].

Group	Body weight [b]	Absolute weights			Relative weights		
		thymus [b]	spleen [b]	PLN [c]	thymus [d]	spleen [d]	PLN [e]
control	219.2 ± 16.1	0.66 ± 0.10	0.55 ± 0.10	15.1 ± 5.0	3.00 ± 0.32	2.52 ± 0.38	68.1 ± 20.0
DOP	201.3 ± 8.5 **	0.37 ± 0.07 ***	0.41 ± 0.04 ***	9.8 ± 3.0 **	1.86 ± 0.38 ***	2.02 ± 0.16 **	48.5 ± 13.1 *
		adrenals [b]	kidneys [b]	liver [b]	adrenals [d]	kidneys [d]	liver [d]
control		0.04 ± 0.01	1.75 ± 0.13	9.45 ± 0.82	0.19 ± 0.03	7.98 ± 0.51	43.10 ± 2.53
DOP		0.04 ± 0.01	1.81 ± 0.22	9.18 ± 0.54	0.21 ± 0.04	8.97 ± 0.82 **	45.60 ± 1.58 *

[a] Rats fed a diet low (2-3 ppm) in pyridoxine were exposed to 40 ppm DOP in drinking water for 15 days. The data are presented as mean ± SD of 10 rats per group and are expressed in g (b), mg (c), g/kg body weight (d), or mg/kg body weight (e). PLN: popliteal lymph nodes.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$, two-tailed Student's *t*-test.

macrophages positive for the monoclonal antibody ED2 (Dijkstra *et al.*, 1985; Beelen *et al.*, 1987) was observed. In the spleen, a decrease in the number of $ER4^+$ (Joling *et al.*, 1985; Vaessen *et al.*, 1985) cells was observed. Like in thymus, the number of macrophages positive for ED2 was decreased in the spleen red pulp. The changes in thymus and spleen could be observed already after 2 days of exposure and appeared maximal at day 7.

Recovery from the effects was apparent within 3 days after withdrawal of THI-exposure, whereas at day 6 of recovery, no differences between control and exposed rats could be noted anymore.

Two-week study on 4'-deoxypyridoxine (DOP). During the first week of exposure, no differences in food intake were noted between control and DOP-exposed rats (data not presented). Between day 7 and day 15 however, DOP-exposed animals took only 86% of the food when compared to the controls. Fluid intake did not differ significantly between the two groups (data not presented). The terminal body weights and absolute and relative organ weights are presented in Table 5.2. Terminal body weights of DOP-exposed rats were significantly decreased when compared to the control weights. Body weights at day 7 did not differ significantly between the two groups (data not presented). Absolute and relative weights of thymus, spleen and popliteal lymph nodes of DOP-exposed rats were all below those of control rats. Absolute weights of adrenals, kidneys and liver were not affected. Whereas the relative adrenal weight was not affected, relative weights of kidneys and liver were slightly increased in DOP-exposed animals.

TABLE 5.3

TOTAL LEUKOCYTE, BLOOD LYMPHOCYTE AND NEUTROPHIL NUMBERS OF CONTROL AND DOP-EXPOSED RATS FED A DIET LOW IN VITAMIN B6 [a].

Group	Total leukocytes	Lymphocytes	Neutrophils
control	14.4 ± 4.7	10.6 ± 4.1	3.6 ± 1.9
DOP	8.1 ± 1.9 **	5.2 ± 1.3 ***	2.7 ± 0.9

[a] Rats fed a diet low (2-3 ppm) in pyridoxine were exposed to 40 ppm DOP in drinking water for 15 days. The data are presented as mean ± SD of 10 rats per group and are expressed in 10^6 cells/ml.

** $p<0.01$; *** $p<0.001$, two-tailed Student's *t*-test.

TABLE 5.4

NUMBERS OF NUCLEATED CELLS ISOLATED FROM VARIOUS LYMPHOID ORGANS OF CONTROL AND DOP-EXPOSED RATS FED A DIET LOW IN VITAMIN B6 [a].

Group	Thymus [b]	Spleen [b]	Bone marrow [b]	PLN [c]
control	18.8 ± 3.1	7.1 ± 1.0	2.3 ± 0.2	6.0 ± 1.8
DOP	7.0 ± 3.3 ***	4.6 ± 0.7 **	2.0 ± 0.3	4.1 ± 2.5

[a] Rats fed a diet low (2-3 ppm) in pyridoxine were exposed to 40 ppm DOP in drinking water for 15 days. The data are presented as mean ± SD of 5 rats per group and are expressed in 10^8 (b) or 10^6 (c) nucleated cells per organ. Numbers of bone marrow and popliteal lymph node (PLN) cells indicate the total number of nucleated cells isolated from both popliteal lymph nodes and femoral bones.

** $p<0.01$; *** $p<0.001$, two-tailed Student's *t*-test.

Results of total leukocyte, blood lymphocyte and neutrophil counts are presented in Table 5.3. Total leukocyte and blood lymphocyte numbers were significantly decreased in DOP-exposed animals. The number of blood neutrophils was not affected. No differences were observed in blood eosinophil, basophil and monocyte numbers (data not presented). Numbers of nucleated cells isolated from lymphoid organs are presented in Table 5.4. Total numbers of nucleated cells in thymi and spleens of DOP-exposed rats were significantly decreased. Total bone marrow and popliteal lymph node cell numbers did not differ significantly between the two groups.

Histopathologic examination of thymic sections stained with hematoxylin and eosin revealed a decrease in cortical area and a relative increase in medullary area in DOP-exposed animals. Moreover, cell densities in the medullary and the cortical area in thymi of DOP-exposed rats were about the same. Pathologic examination of hematoxylin and eosin stained section of spleen, lymph nodes, liver, kidneys and adrenals did not reveal remarkable differences between DOP-exposed and control animals.

Study on time-effect relationship with DOP. At day 2 of exposure, total leukocyte numbers in DOP-exposed animals were decreased to 41% ($p<0.05$) of the control counts. Between day 2 and day 28, total leukocyte counts in the exposed rats fluctuated between 25 and 40% ($p<0.01$) of the control counts.

At immunohistological examination of thymic sections, a strong increase in mainly $CD4^+$, $HIS45^+$ thymocytes was observed in the medulla of DOP-exposed rats. In the cortex of the thymus, a strong decrease in the number of macrophages positive for the monoclonal antibody ED2 was observed. In the spleen, a decrease in the number of $ER4^+$ cells was observed, while the number of macrophages positive for ED2 in the red pulp was decreased also. The changes in thymus and spleen could be observed already after 2 days of exposure and were maximum at day 7.

Ex vivo and in vitro studies on DOP. Results of the *ex vivo* studies with DOP are presented in Table 5.5 (results of one representative experiment with splenocytes) and Table 5.6 (results of one representative experiment with thymocytes). The

TABLE 5.5

SPONTANEOUS AND MITOGEN-INDUCED ^{3}H-THYMIDINE INCORPORATION BY SPLENIC CELLS FROM CONTROL AND DOP-EXPOSED RATS FED A DIET LOW IN VITAMIN B6 [a].

	amount of mitogen per well		
Group	0 µg PHA	25 µg PHA	75 µg PHA
control	0.3 ± 0.2	4.3 ± 0.6	17.9 ± 5.9
DOP	0.3 ± 0.0	1.2 ± 0.5 ***	0.3 ± 0.1 ***
	amount of mitogen per well		
	0 µg Con A	0.2 µg Con A	0.5 µg Con A
control	0.4 ± 0.1	6.2 ± 1.3	14.8 ± 2.7
DOP	0.3 ± 0.0	1.2 ± 0.4 ***	3.4 ± 1.0 **
	amount of mitogen per well		
	0 µg LPS	15 µg LPS	25 µg LPS
control	0.4 ± 0.2	1.5 ± 0.7	1.4 ± 0.7
DOP	0.5 ± 0.4	0.4 ± 0.2 *	0.4 ± 0.1 *

[a] Rats fed a diet low (2-3 ppm) in pyridoxine were exposed to 40 ppm DOP in drinking water for 14 days. The data are presented as the mean ± SD of four animals and are expressed in 10^3 cpm/well.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$, two-tailed Student's *t*-test comparison with controls.

TABLE 5.6

SPONTANEOUS AND MITOGEN-INDUCED ^{3}H-THYMIDINE INCORPORATION BY THYMOCYTES FROM CONTROL AND DOP-EXPOSED RATS FED A DIET LOW IN VITAMIN B6 [a].

	amount of mitogen per well		
group	0 µg PHA	25 µg PHA	75 µg PHA
control	0.1 ± 0.0	0.2 ± 0.1	0.7 ± 0.6
DOP	0.1 ± 0.0	1.2 ± 0.4 **	10.3 ± 4.1 **
	amount of mitogen per well		
	0 µg Con A	0.2 µg Con A	0.5 µg Con A
control	0.1 ± 0.0	10.6 ± 2.6	13.9 ± 4.4
DOP	1.4 ± 2.3	19.4 ± 5.2 *	27.7 ± 4.5 **

[a] Rats fed a diet low (2-3 ppm) in pyridoxine were exposed to 40 ppm DOP in drinking water for 14 days. The data are presented as the mean ± SD of four animals and are expressed in 10^3 cpm/well.

* $p<0.05$; ** $p<0.01$, two-tailed Student's *t*-test comparison with controls.

results demonstrated an impaired response to mitogenic stimulation of splenic cells from DOP-exposed rats. An increase in mitogen-induced thymidine incorporation in thymocytes from DOP-exposed rats was observed in comparison to the controls.

DOP *in vitro* at concentrations up to 1.0 mM did not affect the incorporation of thymidine in splenic cells after stimulation with PHA or in the absence of the mitogen (data not presented).

Discussion

The results presented in this paper indicate that effects of THI and the vitamin B6 antagonist 4'-deoxypyridoxine (DOP) on the rat immune system are quite similar. Both THI and DOP induce a strong and rapid decrease in blood lymphocyte numbers in rats fed a diet containing 2 to 3 ppm vitamin B6. Although the number of blood neutrophils was not affected by exposure to DOP in the current two week study, increased numbers of blood neutrophils in rats fed a diet low in vitamin B6 have been observed in other studies with DOP (Robson and Schwarz, 1975; Houben,

unpublished results). Increased numbers of neutrophils were observed in THI-exposed rats in the current studies on the time-effect relationships. This result agrees with other studies on Caramel Colour III (Evans *et al.*, 1977; Gaunt *et al.*, 1977; Houben, unpublished results) and THI (Sinkeldam *et al.*, 1988; Houben, unpublished results). However, in several studies on Caramel Colour III (Sinkeldam *et al.*, 1988; Chapter 2; Houben, unpublished results) and THI (Gobin *et al.*, 1989; Chapter 2; Houben, unpublished results), blood neutrophil numbers were unaffected. Exposure to DOP did not affect the number of nucleated cells in bone marrow, a finding which agrees with studies on Caramel Colour III and THI (Chapter 2). Popliteal lymph node weights were decreased in DOP exposed rats. Although the number of popliteal lymph node cells was not significantly affected by DOP, they tended to be decreased. Decreased numbers of popliteal lymph node cells have been observed in studies on Caramel Colour III and THI (Chapter 2). Spleen weight and cell number were reduced in DOP-exposed, as well as in THI- and Caramel Colour III-exposed (Chapter 2) rats. Both THI- and DOP-exposure were found to induce a decrease in the number of spleen cells positive for the monoclonal antibodies ER4 , considered as a recent thymus emigrant marker (Joling *et al.*, 1985; Vaessen *et al.*, 1985) and ED2, considered as a macrophage differentiation marker (Dijkstra *et al.*, 1985; Beelen *et al.*, 1987). In more recent studies with rats fed a diet containing 2 to 3 ppm vitamin B6 (Houben, unpublished results), the number of ED2+ cells in spleens from rats exposed to 11.44 ppm THI or 57.2 ppm DOP in drinking water for 14 days was decreased to 3% or less ($p<0.001$) when compared to the control numbers. In those studies, a strong decrease in the number of ED2+ cells was also observed in livers of DOP- and THI-exposed rats. In the 2 week study, DOP-exposure induced a decrease in absolute and relative thymus weight. In several short-term studies with Caramel Colour III- and THI-exposure for less than 4 weeks (Chapter 2; Sinkeldam *et al.*, 1988), relative thymus weights were not found to be changed. However, results on relative thymus weights reported from a 13-week study demonstrated a reduction after exposure to Caramel Colour III (Gaunt *et al.*, 1977). The decrease in thymus weight induced by DOP was accompanied by a decrease in the number of nucleated cells in thymus, whereas thymus cell counts in general are not affected by Caramel Colour III or THI within 4 weeks of exposure (Chapter 2; Houben, unpublished results). Differences in the spectrum of disturbed processes may (in part) account for the difference in (the time dependence of) effects on thymus between Caramel Colour III or THI and DOP. Despite the difference in (the time-dependence of) effects

on thymus weight and cell number induced by DOP and Caramel Colour III or THI, similar morphological changes were observed. A decrease in the cortex/medulla area ratio and an increase in medullary cell density were induced both by DOP and THI within 2 to 7 days of exposure, while changes observed in thymus at immunohistological examination were also similar. Moreover, all effects of THI and DOP studied in time occurred rapidly. The effects could be noted already after 2 days of exposure and were about maximum at day 7.

Ex vivo mitogenic stimulation studies demonstrated an impaired response to mitogenic stimulation of splenic cells and an increase in mitogen-stimulated thymidine incorporation in cells from the thymus gland of DOP-exposed as well as THI-exposed (Chaptere 4) rats. These results also indicated that effects of THI and DOP on the rat immune system are similar. DOP did not affect the response to mitogenic stimulation of spleen cells in an *in vitro* study. Although the results from this *in vitro* study were also similar to observations from *in vitro* studies with THI (Chapter 4), the absence of an *in vitro* effect of DOP is somewhat surprising, since the response of human lymphocytes to mitogenic stimulation has been reported to be inhibited by DOP in *in vitro* studies (Scountzou *et al.*, 1989; Goede, 1989). Currently, studies are in progress to examine this discrepancy. Previously (Chapter 2), we suggested that THI disturbs a pyridoxal 5'-phosphate (PLP)-dependent process. Because of the similarity in effects of DOP and THI on the immune system, we now propose that DOP and THI share a same mechanism of immunomodulation.

DOP is an analog of vitamin B6 that is phosphorylated by pyridoxal kinase into the metabolite 4'-deoxypyridoxine 5'-phosphate (DOP-P) (Friedrich, 1988). Due to this phosphorylation, DOP causes a competitive inhibition of the phosphorylation of PLP precursors by pyridoxal kinase, which may result in a decrease in PLP levels in DOP-exposed animals (Sinkeldam *et al.*, 1988). PLP is the major metabolically active form of vitamin B6 and plays an essential role as a cofactor of a large number of enzymes, including transaminases and decarboxylases (Friedrich, 1988). A decrease in PLP levels is generally considered to account for the vitamin B6 antagonising action of DOP. However, effects of DOP on rat lymphocyte counts were reported at doses that did not affect plasma PLP levels (Sinkeldam *et al.*, 1988), while in rats fed a diet low in vitamin B6, exposure to DOP does not necessarily induce a decrease in tissue PLP levels but may even increase tissue PLP contents (Johnston *et al.*, 1966; Coburn *et al.*, 1981). In addition, THI was found to have no effect on PLP levels in rats (Sinkeldam *et al.*, 1988; Chapter 2).

However, DOP has an additional mechanism of vitamin B6 antagonism. As already indicated, PLP is the major active form of vitamin B6. PLP remains bound to the PLP-dependent enzymes, in which the ring nitrogen, the 3'-hydroxyl and the negatively charged 5'-phosphate group play an essential role (Morino and Nagashima, 1984; Snell, 1970). The 4'-aldehyde group of the coenzyme is the active group in the coenzymatic reactions (Friedrich, 1988), but plays no essential role in apoenzyme-binding (Snell, 1970). DOP is metabolized into DOP-P, and because of its structural analogy, the formed DOP-P competes with PLP for binding to the apoenzymes. Since DOP-P has no 4'-aldehyde group and can not act as a coenzyme, this competition with PLP gives rise to an additional mechanism of inhibition of vitamin B6-dependent processes. This inhibition does not require a depletion of PLP, but causes a direct and rapid inhibition of for instance decarboxylating or transaminating enzymes. Several pyridoxal analogs modified in position 4 or in positions 4 and 5 have been found to inhibit pyridoxal kinase (Korytnyk, 1979), while their 5'-phosphorylated metabolites bind to PLP-dependent apoenzymes (Korytnyk, 1979; Yang and Metzler, 1979; Misharin *et al.*, 1979). The analog 4'-*N*-(2,4-dinitro-5-fluorophenyl)-pyridoxamine 5'-phosphate is known to bind to the coenzyme binding site of PLP-dependent apoenzymes (Riva *et al.*, 1979). Moreover, 4'-deoxypyridoxine 5'-phosphate was reported to be a competitive inhibitor of the activation of glutamate apodecarboxylase by PLP (Martin *et al.*, 1990), and in *in vitro* studies it was demonstrated that ornithine decarboxylase activity is inhibited by DOP-P but not by DOP (Conner and Lowe, 1983). Because effects of DOP on the immune system may not necessarily depend on a decrease in PLP levels, we now propose that DOP does not cause immunomodulation primary by the competitive inhibition of pyridoxal kinase. We suggest that effects of DOP on the immune system are mainly caused by a direct inhibition of PLP-dependent enzymes by DOP-P, due to a competition between DOP-P and PLP for binding to the cofactor binding-site of the apoenzymes. Decreased levels of PLP caused by a competitive inhibition of the phosphorylation of PLP-precursors may shift the competition between DOP-P and PLP in favour of DOP-P and may contribute to the inhibition of PLP-dependent enzymes. In addition, considering the similarity in effects of THI and DOP on the rat immune system and the protective potency of vitamin B6 on the THI-induced effects, we propose that THI shares the main mechanism of immunomodulation of DOP and causes its effect on the immune system by a direct competitive inhibition of one or more PLP-dependent enzymes.

Although information on the role of vitamin B6-dependent enzymes in immune processes is far from complete, ornithine decarboxylase is known for its importance in immune processes (Endo *et al.*, 1988; Farrar *et al.*, 1988; Fidelus *et al.*, 1984; Kierszenbaum *et al.*, 1987; Schall *et al.*, 1991), while vitamin B6-dependent enzymatic processes are also required for the production of mediators like histamin and serotonin (Friedrich, 1988). Since vitamin B6 plays a role in many metabolic processes (Friedrich, 1988), it is likely that many more vitamin B6-dependent processes are important to the immune system. Although potentially all enzymatic reactions in which PLP acts as a coenzyme may be suppressed due to the proposed action of DOP-P, and possibly THI, the effects of THI and DOP on the immune system may likely result from interference with one or several sensitive enzymes or processes. Differences in affinity of binding to the cofactor binding site of the apoenzymes (Korytnyk *et al.*, 1976; Snell, 1970) may contribute to this selectivity. Furthermore, differences in affinity may also result in a different spectrum of inhibition of vitamin B6 dependent enzymes by THI, in comparison to vitamin B6 deficiency or DOP-exposure. The latter may explain why classical signs of a vitamin B6 deficiency, which may also be induced by DOP, were never observed in toxicity studies with Caramel Colour III and THI.

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Chapter 6

Effects of the colour additive Caramel Colour III on the immune system of mice

Geert F. Houben[1,2], Willem Seinen[1], and André H. Penninks[1,2]

1: Research Institute of Toxicology, Department of Immunotoxicology, University of Utrecht, Utrecht, The Netherlands
2: TNO Toxicology and Nutrition Institute, Zeist, The Netherlands

Abstract

Administration of the colour additive Caramel Colour III has been observed to induce a reduction in blood lymphocyte counts in rats. Beside lymphopenia, several other changes in lymphoid tissues as well as changes in immune function parameters have been observed. The effects are caused by the imidazole-derivative 2-acetyl-4(5)-tetrahydroxybutyl-imidazole (THI), and occur especially in animals fed a diet low in vitamin B6. There is evidence indicating that the effects of Caramel Colour III and THI in rats are caused by a change in activity of macrophages. Because information on the mouse immune system is more detailed and more experimental models and determination techniques are available for the mouse, the mouse possibly provides a more suitable species than the rat for future mechanistic studies on the effects of Caramel Colour III and THI on the immune system. Therefore, effects of Caramel Colour III on several parameters of the immune system were studied in mice fed a diet containing 2 to 3 ppm vitamin B6 and the effects were compared with those observed in prior studies in rats. In addition, a dose-range finding was carried out. The results demonstrated that Caramel Colour III induces similar effects in mice as it does in rats, thereby justifying the choice of the mouse as a species for mechanistic studies. However, the results indicated that the doses required to obtain an equivalent effect in mice are somewhat higher. Therefore, the use of synthetic THI must be preferred to avoid interference of Caramel Colour III-administration with food and/or water consumption.

Introduction

Caramel Colours (E150) are among the oldest and most commonly used colour additives in human food stuffs and drinks. Their use accounts for at least 95% by weight of the permitted colour additives used in food. Based on the reactants used for their production, commercial Caramel Colours have been classified into four classes. Among these, the use of Class III Caramel Colour, also known as Ammonia Caramel, accounts for 20 to 25% of the total use of Caramel Colours in the USA, and for about 50% in Europe.

Administration of Caramel Colour III has been observed to induce a reduction in blood lymphocyte counts in rats (Evans *et al.*, 1977; Gaunt *et al.*, 1977; Noltes and Chappel, 1987; Sinkeldam *et al.*, 1988; Chapter 2). Beside lymphopenia, reduced

numbers of nucleated cells in peripheral lymphoid organs, changes in thymus morphology and changes in macrophage subpopulations as well as in lymphocyte subset distribution have been observed (Chapter 2). Moreover, immune function tests demonstrated a suppressed thymus-dependent immunity, while parameters of the non specific resistance are also affected, as shown by a reduced NK activity in the spleen and an enhanced clearance of *Listeria monocytogenes* (Chapter 3). In *ex vivo* studies with splenic cells from Caramel Colour III-exposed rats, an impaired response to mitogenic stimulation, presumably due to a suppressive activity of adherent cells, was observed (Chapter 4). The effects of Caramel Colour III are caused by 2-acetyl-4(5)-tetrahydroxybutyl-imidazole (THI) (Chapter 2; Kröplien *et al.*, 1985; Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988), and occur especially in animals fed a diet low in vitamin B6 (Chapter 2; Gobin and Paine, 1989; Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988).

Previously, we suggested that the effects of Caramel Colour III and THI are caused by a change in activity of macrophages (Chapters 2, 3 and 4). Therefore, part of future mechanistic studies on the effects of Caramel Colour III and THI on the immune system may concentrate on macrophage functions, in particular on phagocytic and killing activity, antigen processing and presentation and interleukin production. For some of future studies, the mouse may provide a more suitable species than the rat because information on the mouse immune system is more detailed, and more experimental models and determination techniques are available. To investigate the suitability of the mouse as a species for mechanistic studies on Caramel Colour III- or THI-induced changes in the immune system, effects of Caramel Colour III on several parameters of the immune system were studied in mice fed a diet containing 2 to 3 ppm vitamin B6, and the effects were compared with those observed in rats. In addition, a dose-range finding was carried out.

Materials and Methods

Materials. Caramel Colour III was manufactured by Hay-Lambert Ltd (Uxbridge, Middx, UK) and was provided by the International Technical Caramel Association (ITCA, Washington, DC, USA). The chemical specifications of the Caramel Colour III, batch 2422, have been reported before (Sinkeldam *et al.*, 1988). The 2-acetyl-4(5)-tetrahydroxybutyl-imidazole (THI) content of Caramel Colour III batch 2422 was about 200 ppm.

Animals and maintenance. Male mice from an outbred strain (CDI-Swiss; Charles River Laboratories, France S.A.) were used in these studies. At the start of the study, the animals had a body weight of 30.8 ± 1.4 g. The animals were housed individually in makrolon boxes on a bedding of chips of wood in an animal room kept at a temperature of 22-24 °C with a relative humidity of 40-60%, and a 12-hr light/dark cycle. Semi-purified casein diet (TNO Toxicology and Nutrition Institute, Zeist, The Netherlands) containing 2 to 3 mg vitamin B6 per kg (vitamin B6 supplemented as pyridoxine·HCl, Merck, Darmstadt, FRG) and slightly acidified demineralized water (with HCl to pH 3.0) whether or not supplemented with Caramel Colour III were provided *ad libitum*.

Experimental design and conduct. Groups of mice were provided either with acidified demineralized water (controls), or a 1, 4 or 8% (w/w) Caramel Colour III solution (equal to THI solutions of about 2, 4 and 16 ppm, respectively). The Caramel Colour III-exposure was continued for 14 days. The individual body weights of the mice were recorded at days 0, 7 and 14 of the study. After an experimental period of 14 days, the animals were anaesthetized with ether and blood samples were taken by orbital puncture. The blood was used for the determination of total white blood cell counts using a Coulter Counter model ZF (Coulter Electronics Ltd., Harpender, Herts, UK) and for preparation of blood smears. An estimation of the relative numbers of the different types of leukocytes was obtained by counting 100 cells in the smears after Pappenheim staining (Gorter and de Graaf, 1955). From the total and the differential leukocyte counts, the absolute numbers of the different types of leukocytes were calculated. Blood samples from control and 8% Caramel Colour III-exposed animals were also used for the determination of plasma pyridoxal 5'-phosphate levels according to a method modified after Chabner and Livingstone (1970). In addition, blood lymphocytes from control and 8% Caramel Colour III-exposed mice were enriched by Ficoll-Paque (Pharmacia Fine Chemicals AB, Uppsala, Sweden) centrifugation following the manufacturers instructions. After fixation in 0.5% paraformaldehyde for 5 minutes, the cells were resuspended in phosphate buffered saline. Lymphocytes were stained by an immunoperoxidase procedure on cytospin centrifugation preparations using appropriate dilutions of rat anti-mouse monoclonal antibodies. T lymphocytes were stained using anti-Thy 1 (Ledbetter and Herzenberg, 1979) and B cells using anti-B220 (Coffman and Weissmann, 1981) (antibodies obtained through Medigon from Dr. v. Ewijk, Erasmus University Rotterdam, The Netherlands). The preparations were stained for

peroxidase in DAB and H_2O_2. Subsequently, the cells were stained with hematoxylin. A total of 100 cells was counted and the B over T ratios were calculated. After collection of the blood, the mice were killed by decapitation. At necropsy, weights of thymus and spleen were recorded. Parts from thymus and spleen were embedded in plastic after fixation in 4% phosphate-buffered formaldehyde. Sections stained with hematoxylin and eosin were examined by light microscopy. An estimation of the relative cortical area in thymus and the number of megakaryocytes in spleen was obtained by morphometrical analysis (Mini Mop, Kontron Bildanalyse, Munich, FRG) of serial sections stained with hematoxylin and eosin.

Statistical analysis. The results were analysed for significance of differences by anova & LSD test. Data were considered significant when $p<0.05$.

Results

Body weights and organ weights. Results on body weight determinations are presented in Table 6.1. After 7 and 14 days of exposure to various concentrations of

TABLE 6.1

BODY WEIGHTS ON DAYS 0, 7 AND 14 AND TOTAL BODY WEIGHT-GAIN OF CONTROL- AND 1, 4, OR 8% CARAMEL COLOUR III-EXPOSED MICE FED A DIET CONTAINING 2 TO 3 PPM VITAMIN B6.

group	body weight			total body weight gain
	day 0	day 7	day 14	
control	30.9 ± 0.9	32.6 ± 1.6	33.9 ± 1.2	3.1 ± 0.6 (10.0%)
1%	30.7 ± 0.7	32.1 ± 2.4	33.5 ± 2.6	2.9 ± 2.2 (9.3%)
4%	31.1 ± 1.5	31.6 ± 1.6	32.3 ± 1.5	1.3 ± 1.1 (4.2%)
8%	30.5 ± 2.3	30.8 ± 2.3	30.9 ± 3.4	0.4 ± 1.4 (1.2%) *

Male Swiss mice were exposed to Caramel Colour III in drinking water for 14 days. The Caramel Colour III batch used contained 200 ppm THI. Body weights and body weight gain are presented in g and as mean ± SD of 5 animals per group. Mean body weight gain is also expressed as a percentage of the body weight at day 0.

* $p<0.01$ vs. control group; $p<0.05$ vs. 1% Caramel Colour III group (anova & LSD test).

Caramel Colour III in drinking water, no significant differences in mean body weights were observed. However, mice provided with 8% Caramel Colour III gained significantly less weight during the 14-days experimental period. Mean body weight gain over the period of day 0 to day 7 did not differ significantly among the four groups (data not presented). Absolute and relative weights of thymus and spleen are presented in Table 6.2. There were no significant differences in absolute and relative weights of these organs associated with exposure to Caramel Colour III.

Blood cell parameters. Results of total leukocyte, blood lymphocyte and blood neutrophil counts are presented in Table 6.3. A dose-dependent decrease in mean total leukocyte and blood lymphocyte numbers was observed, reaching the level of statistical significance for 8% Caramel Colour III-exposed animals. In these animals, total leukocyte and blood lymphocyte numbers were decreased to 45 and 32% of control numbers, respectively. Blood neutrophil numbers were unaffected by exposure to Caramel Colour III. Blood eosinophil, basophil and monocyte numbers were not affected either (data not presented). The mean B cell/T cell ratios in 8% Caramel Colour III-exposed mice (0.84 ± 0.38) did not differ significantly from those in control mice (0.25 ± 0.02).

TABLE 6.2

ABSOLUTE AND RELATIVE WEIGHTS OF THYMUS AND SPLEEN OF CONTROL- AND 1, 4, OR 8% CARAMEL COLOUR III-EXPOSED MICE FED A DIET CONTAINING 2 TO 3 PPM VITAMIN B6.

group	absolute organ weights		relative organ weights	
	thymus	spleen	thymus	spleen
control	0.034 ± 0.008	0.089 ± 0.014	1.0 ± 0.2	2.6 ± 0.4
1%	0.037 ± 0.010	0.102 ± 0.028	1.1 ± 0.3	3.0 ± 0.6
4%	0.041 ± 0.015	0.089 ± 0.011	1.2 ± 0.4	2.8 ± 0.4
8%	0.026 ± 0.010	0.072 ± 0.014	0.8 ± 0.3	2.3 ± 0.4

Male Swiss mice were exposed to Caramel Colour III in drinking water for 14 days. The Caramel Colour III batch used contained 200 ppm THI. Organ weights are presented in g or in g/kg body weight and as mean ± SD of 5 animals per group.

TABLE 6.3

TOTAL WHITE BLOOD CELL, BLOOD LYMPHOCYTE AND NEUTROPHIL NUMBERS IN CONTROL- AND 1, 4, OR 8% CARAMEL COLOUR III-EXPOSED MICE FED A DIET CONTAINING 2 TO 3 PPM VITAMIN B6.

group	total leukocytes	lymphocytes	neutrophils
control	6.7 ± 1.7	5.6 ± 1.8	1.2 ± 0.5
1%	6.7 ± 2.9	5.3 ± 1.5	1.3 ± 1.5
4%	5.5 ± 1.0	4.5 ± 0.4	1.0 ± 0.7
8% #	3.0 ± 0.5 *	1.8 ± 0.2 °	1.3 ± 0.7

Male Swiss mice were exposed to Caramel Colour III in drinking water for 14 days. The Caramel Colour III batch used contained 200 ppm THI. Blood cell numbers are presented in 10^9/l and as mean ± SD of 5 or 4# animals per group.

* $p<0.01$ vs. control and 1% Caramel Colour III group; $p<0.05$ vs. 4% Caramel Colour III group (anova & LSD test).

° $p<0.001$ vs. control and 1% Caramel Colour III group; $p<0.01$ vs. 4% Caramel Colour III group (anova & LSD test).

Histological observations. Results of cortical area measurements in thymus sections are presented in Table 6.4. The relative cortical area in 8% Caramel Colour III-exposed mice was decreased significantly. From histological examination of thymus sections from these animals, there appeared to be a slight increase in medullary cell density. Other abnormalities were not observed in thymi of Caramel Colour III-exposed mice. Although the number of megakaryocytes in spleens of Caramel Colour III-exposed mice tended to be decreased, there were no statistically significant differences in relative megakaryocyte numbers between the four groups (Table 6.4). Histological examination of spleen sections did not reveal remarkable differences between control and exposed animals.

Plasma pyridoxal 5'-phosphate levels. Plasma pyridoxal 5'-phosphate levels in control mice (388.4 ± 45.9 nmol/l) did not differ significantly from those in 8% Caramel Colour III-exposed animals (369.4 ± 31.5 nmol/l).

TABLE 6.4

RELATIVE CORTICAL AREA IN THYMI AND RELATIVE MEGAKARYOCYTE NUMBERS IN SPLEENS OF CONTROL- AND 1, 4, OR 8% CARAMEL COLOUR III-EXPOSED MICE FED A DIET CONTAINING 2 TO 3 PPM VITAMIN B6.

group	relative cortical area	megakaryocyte number
control	63 ± 7	100 ± 54
1%	65 ± 7	83 ± 47
4%	66 ± 5	96 ± 48
8%	50 ± 10 *	61 ± 33

Male Swiss mice were exposed to Caramel Colour III in drinking water for 14 days. The Caramel Colour III batch used contained 200 ppm THI. The relative cortical area in thymi is expressed in cortical area as a percentage of the total area. The relative numbers of megakaryocytes in spleens are presented as a percentage of the controls. The data are presented as mean ± SD of 5 animals per group.

* $p<0.01$ vs. 1 and 4% Caramel Colour III group; $p<0.05$ vs. control group (anova & LSD test).

Discussion

The objectives of this study were to investigate whether Caramel Colour III induces similar changes in the mouse immune system as it does in rats, and to get an impression of the doses of Caramel Colour III (more specifically: of 2-acetyl-4(5)-tetrahydroxybutyl-imidazole, THI) required. Therefore, several of the parameters studied previously in Caramel Colour III or THI studies with rats were determined in control mice and mice exposed to 1, 4 or 8% Caramel Colour III in drinking water for 14 days.

The results indicate that Caramel Colour III induces changes in the immune system of mice fed a diet containing 2 to 3 mg vitamin B6/kg, similar to those observed in rats. As in Caramel Colour III and THI studies with rats (Evans *et al.*, 1977; Gaunt *et al.*, 1977; Conway and Paine, 1986a,b,c; Conway and Paine, 1988; Sinkeldam *et al.*, 1988; Gobin and Paine, 1989; Gobin *et al.*, 1989; Chapter 2), a decrease in total leukocyte and blood lymphocyte numbers was induced, due to a

reduction in B as well as in T cells. Blood neutrophil numbers were unaffected, which is in accordance with several rat studies on Caramel Colour III (Sinkeldam *et al.*, 1988; Chapter 2; Houben, unpublished results) and THI (Gobin *et al.*, 1989; Chapter 2; Houben, unpublished results). However, increased numbers of blood neutrophils have been observed in other studies with Caramel Colour III or THI in rats (Evans *et al.*, 1977; Gaunt *et al.*, 1977; Sinkeldam *et al.*, 1988; Chapter 5; Houben, unpublished results). As in rat studies (Chapter 2), blood eosinophil, basophil and monocyte numbers were unaffected. Absolute and relative thymus weights were not affected either, which is in accordance with observations from several short-term studies with Caramel Colour III- or THI-exposure of rats for 28 days or less (Sinkeldam *et al.*, 1988; Chapter 2). No significant differences in absolute or relative spleen weights were observed in this mouse study, which is in agreement with several rat studies that failed to demonstrate an effect of Caramel Colour III or THI on relative (Evans *et al.*, 1977; Gaunt *et al.*, 1977; Houben, unpublished results) or absolute (Houben, unpublished results) spleen weight. However, reduced absolute (Gaunt *et al.*, 1977; Chapter 2) and relative (Sinkeldam *et al.*, 1988; Chapter 2) spleen weights have been observed in other rat studies. Although absolute and relative thymus weights were not affected, a decrease in relative cortical area was observed in thymi of exposed mice, an effects that was also observed in rats (Chapter 2). As in rats also (Chapter 2), medullary cell density was slightly increased. An increase in the number of megakaryocytes that was reported from a rat study (Chapter 2) was not observed in this mouse study. Because there is some evidence indicating that Caramel Colour III may affect mammalian vitamin B6 metabolism (Spector and Huntoon, 1982; Schreurs and Sinkeldam, 1985; Sinkeldam *et al.*, 1988), parameters of the vitamin B6 status have been determined in several rat studies. The results indicated that Caramel Colour III-exposure is not always accompanied by changes in the vitamin B6 status in rats (Conway and Paine, 1986a; Houben, unpublished results). In the current mouse study, plasma pyridoxal 5'-phosphate levels were not changed upon exposure to 8% Caramel Colour III.

The similarity in effects of Caramel Colour III in mice and rats indicates that the mouse may be a suitable species for future mechanistic studies. However, although a detailed dose-effect relationship has not been established yet, these preliminary data suggest that the doses required to obtain an equivalent effect in mice as in rats are somewhat higher. Previously, 200 ppm THI in drinking water has been reported to induce lymphopenia in male BALB/c mice, whereas 100 ppm was inactive (Iscaro *et*

al., 1988). However, the vitamin B6 content of the diet in that mouse study was 33 mg/kg, which is far above the defined level of 1 mg/kg required for normal growth and reproduction in mice (National Academy of Sciences, 1978). In particular because of the known interference of the vitamin B6 status with Caramel Colour III- and THI-induced effects in rats (Chapter 2; Gobin and Paine, 1989; Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988), results obtained in a study with mice fed a diet containing 33 mg vitamin B6/kg do not allow the conclusion that mice are much less susceptible to THI-induced lymphopenia than rats (Iscaro *et al.*, 1988) and do not pre-exclude the use of mice for mechanistic studies on THI. In the current study with mice fed a diet containing 2 to 3 mg vitamin B6/kg, statistically significant effects were observed upon exposure to 8% Caramel Colour III in drinking water. With the THI-content of the Caramel Colour III batch used of 200 ppm, this 8% solution of Caramel Colour III contained 16 ppm THI. Based on an assumed liquid intake of 200 g/kg body weight/day, the mice provided with 8% Caramel Colour III had an oral intake of THI of 3.2 mg/kg body weight/day. In mice exposed to 4% Caramel Colour III (estimated THI-intake: 1.6 mg/kg body weight/day), no statistically significant effects were determined. In rats, on the other hand, lymphopenia has been reported to occur within a few days upon a THI-intake estimated as low as 20 μg/kg body weight/day (Sinkeldam *et al.*, 1988). Most other effects in rats have been observed in studies with a THI-intake between 0.5 and 0.8 mg/kg body weight/day (Chapter 2), and were also found to occur within 7 days of exposure (Chapter 5). These data indicate that on a mg/kg body weights basis, mice are less sensitive to THI-induced changes than rats. Differences in vitamin B6 dependence may (in part) account for the difference in sensitivity between mice and rats. Therefore, vitamin B6 dependence of the effects of Caramel Colour III or THI on the immune system of mice will have to be studied prior to mechanistic studies using that species. However, differences in the toxicokinetics (absorption, distribution, metabolism and/or excretion) of THI or differences in sensitivity of the immune system may also (in part) account for the difference in sensitivity between mice and rats. With respect to the toxicokinetics and possible metabolism of THI, studies in rats indicated that THI is not metabolised but is excreted rapidly in the urine (Houben, unpublished results; Phillips and Paine, 1990). Nevertheless, possible metabolism of THI may be subject to inter-species differences.

Although there were no significant differences in mean body weights between the four groups, mice provided with 8% Caramel Colour III gained significantly less

weight during the 14-day experimental period. A decrease in body weight gain has also been observed in some studies with rats at high doses of Caramel Colour III (Houben, unpublished results), and is likely to be related to a reduction in food and/or liquid intake. Since the doses of THI required to obtain an equivalent effect in mice may be somewhat higher, the use of synthetic THI (Sweeny *et al.*, 1985) in future studies must be preferred to avoid interference of Caramel Colour III-intake with food and/or water consumption. Moreover, because every batch of Caramel Colour III is an unique and complex mixture of mainly unidentified chemical compounds (Patey *et al.*, 1985a,b; Patey *et al.*, 1987), the use of pure THI has to be preferred above a whole Caramel Colour III batch in mechanistic studies.

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Chapter 7

Effects of the colour additive Caramel Colour III on the immune system; a study with human volunteers

Geert F. Houben[1,2,3], Henk van den Berg[1], Wim van Dokkum[1], Henk van Loveren[3], André H. Penninks[1,2], Willem Seinen[2], Steven Spanhaak[1], Joseph G. Vos[3], and Theo Ockhuizen[1]

1: TNO Toxicology and Nutrition Institute, Zeist, The Netherlands
2: Research Institute of Toxicology, Department of Immunotoxicology, University of Utrecht, Utrecht, The Netherlands
3: National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands

Abstract

Administration of the colour additive Caramel Colour III to rats has been associated with decreased numbers of lymphocytes and several other changes in the immune system as well as in immune function parameters, especially in animals fed a diet with a relatively low content of vitamin B6. The effects are caused by 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI). Caramel Colour III is commonly used in food products such as bakery products, soya-bean sauces, brown sauces, gravies, soup aromas, brown (dehydrated) soups, brown malt caramel blend for various applications, vinegars and beers, and effects in humans upon dietary intake can not be excluded. Biochemically marginally vitamin B6 deficient elderly male volunteers were considered a relevant and potentially sensitive group to study possible effects of Caramel Colour III on blood lymphocyte numbers (total and within subsets) or on proliferative responses of lymphocytes to mitogenic stimulation. In addition, several other haematological parameters as well as serum immunoglobulin levels and *in vitro* immunoglobulin production by Pokeweed mitogen-stimulated mononuclear blood cells were studied. The results of this double-blind intervention study demonstrated that in a selected test group of apparently healthy elderly male volunteers with a biochemically marginally deficient vitamin B6 status, Caramel Colour III containing 23 (commercial sample) or 143 (research sample) ppm THI and administered at the level of the current Acceptable-Daily-Intake (ADI) of 200 mg/kg body weight/day during 7 days did not affect any of the parameters evaluated.

Introduction

Administration of the colour additive Caramel Colour III (Ammonia Caramel Colour) has been associated with decreased numbers of blood lymphocytes in rats (Evans *et al.*, 1977; Gaunt *et al.*, 1977; Noltes and Chappel, 1985; Sinkeldam *et al.*, 1988; Chapter 2), especially in animals fed a diet with a relatively low content of vitamin B6 (Sinkeldam *et al.*, 1988; Gobin and Paine, 1989; Chapter 2). Beside lymphopenia, reduced numbers of nucleated cells in peripheral lymphoid organs, changes in thymus morphology and changes in macrophage subpopulations as well as in lymphocyte subset distribution were observed (Chapter 2). Moreover, changes in immune function parameters were demonstrated, among which a severe impairment

in the response to mitogenic stimulation of splenic cells from exposed rats (Chapters 3 and 4). The effects are caused by the imidazole-derivative 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI) (Kröplien *et al.*, 1985; Sinkeldam *et al.*, 1988; Chapter 2).

Ammonia Caramel Colours constitute a class of Caramel Colour commonly used in food products, including various bakery products, soya-bean sauces, brown sauces, gravies, soup aromas, brown (dehydrated) soups, brown malt caramel blend for various applications, vinegars and beers, particularly in certain dark-brown beers. Their use accounts for 20 to 25% of the total use of Caramel Colours in the USA and for about 50% in Europe. Effects in (marginally vitamin B6 deficient) humans upon dietary intake of Caramel Colour III can not be excluded. A recent survey among the Dutch population demonstrated a frequently occurring biochemically marginally vitamin B6 deficiency in males at an age of 65 or more (Löwik *et al.*, 1989; Bode and Van Den Berg, 1990). Because of the observed influence of the vitamin B6 status on the Caramel Colour III- and THI-induced effects in rats, biochemically marginally vitamin B6 deficient elderly males were considered a relevant and potentially sensitive group to study possible effects of Caramel Colour III in humans. The main goals of the human study were to investigate whether oral intake of Caramel Colour III at the current Acceptable-Daily-Intake (ADI) for 7 days (1) causes a change in the number of blood lymphocytes in human volunteers with a biochemically marginally deficient vitamin B6 status and (2) induces changes in lymphocyte subset ratios or in proliferative responses of lymphocytes to mitogenic stimulation. In addition, several other parameters were studied. Two batches of Caramel Colour III were tested: one commercial batch with a THI-content of 23 mg/kg and one batch with an elevated THI-content of 143 mg/kg.

Materials and methods

Test group, volunteer recruitment and selection. A recent survey among the Dutch population demonstrated a frequently occurring biochemically marginally vitamin B6 deficiency in males at an age of 65 or more (Löwik *et al.*, 1989; Bode and Van Den Berg, 1990). Because of the observed influence of the vitamin B6 status on the Caramel Colour III- and THI-induced effects in rats (Sinkeldam *et al.*, 1988; Gobin and Paine, 1989; Chapter 2), the biochemically marginally vitamin B6 deficient elderly males were considered to constitute a relevant and potentially sensitive

group to study possible effects of Caramel Colour III in humans. By means of an advertisement in local papers, healthy males aged 65 or more were asked to volunteer. Based on a dietary habit and life style analysis revealed by using a structured questionnaire, haematology, clinical chemistry and a standard medical examination, apparently healthy subjects with regular Dutch dietary habits and life style were selected. Since vitamin B6 deficiency in humans is known to be associated with decreased plasma pyridoxal 5'-phosphate (PLP) levels and with increased cofactor stimulation of aspartate aminotransferase in erythrocytes (α-EAST) (Löwik *et al.*, 1989; Van Den Berg *et al.*, 1988), these parameters were used to identify and select biochemically marginally vitamin B6 deficient subjects. Based on these parameters, about 34% of the volunteers had a good vitamin B6 status (plasma PLP ≥ 35 nmol/l and/or α-EAST ≤ 1.69) and were excluded from participation. Finally, from 27 remaining subjects, 24 participants were designated at random. Apart from a planned vitamin B6 supplementation at the termination of the study, none of the participants used any vitamin B6 containing preparations for at least 14 days prior to, and during the study.

Study design and Parameters. During the study, all volunteers lived at home and consumed their regular diets. However, products with a high content of vitamin B6, as well as products containing Caramel Colour III were avoided by using a dietary instruction list. The study was preceded by a 14 day control period during which the subjects adhered to the dietary instructions. This period was included to stabilize the individual vitamin B6 status, to eliminate a possible influence of a previous Caramel Colour III intake and to determine the baseline values (days -14, -7 and 0) of the blood parameters to be studied. The 24 subjects were allocated into 3 groups (one control group and two test groups) of 8 individuals each by computer randomization and proportionately to days -14 and -7 blood lymphocyte numbers and vitamin B6 status (plasma PLP level and α-EAST). Starting at day 0, all persons consumed a dessert twice a day for 7 days. The persons in the control group had a dessert without Caramel Colour III while each of the desserts of the other groups contained half of the current Acceptable-Daily-Intake (current ADI: 200 mg/kg body weight/day) of a Caramel Colour III. Blood samples were taken at day 3 and day 7. The experiment was terminated after a recovery of 26 days during which 4 additional test points were incorporated (days 10, 14, 21 and 33). From day 21 until day 33, all participants could use a vitamin B6 supplementation. At the days indicated, the subjects visited the TNO Toxicology and Nutrition Institute between 9

and 10 a.m. for blood sampling. The blood samples were used for haematological evaluation and to measure parameters of the vitamin B6 status. In addition, cortisol and immunoglobulin levels were measured. Lymphocyte subset numbers were determined by means of flow cytometric analysis and mitogenic response assays were conducted. To screen for the occurrence of an infection, the acute phase proteins C-reactive protein (CRP) and α1-antitrypsin were determined. The study was conducted according to a double-blind protocol. The research proposal and study design were approved by an independent ethical committee and informed consent was obtained from all test persons.

Desserts. The desserts were prepared from vanilla custard, whipping-cream and white moist sugar. All persons consumed a portion of dessert twice a day. The portions were determined on the basis of individual body weights, so that each portion for the persons in the test groups contained half the current ADI. To establish this, the individual body weights were grouped into classes of 5 kg difference (60-65 up to 90-95 kg) and the dose of dessert was based on the upper limit of the class-weight. The amount of Caramel Colour III was dosed to the nearest gram (1 g/ 5 kg body weight). Two batches of Caramel Colour III were tested: one with a THI content of 23 ppm and the other containing 143 ppm of this imidazole-derivative. The test-desserts contained 42.3 g Caramel Colour III per 1000 g. Persons in the control group consumed a dessert that contained no Caramel Colour III, but to which coffee powder ("Moccona" roodmerk, Douwe Egberts, Utrecht, The Netherlands) and Cocoa (van Houten, Hoofddorp, The Netherlands) were added for colour and taste. These products contained no Caramel Colour III. The desserts were prepared according to standardised procedures. Per test group, dessert was prepared as one batch, no longer than 3 days before consumption. The desserts were stored cooled (4 to 7° C) and in dark until use.

Caramel Colour III. The Ammonia Caramel Colours were provided by the International Technical Caramel Association (ITCA, Washington DC, USA). The specifications of the batches were as follows. Batch NA 1098 (Cerestar UK Ltd, Manchester, UK): K 610 (absorbance at 610 nm (path length 1 cm) of an aqueous 0.1% (w/v) solution of Caramel Colour III): 0.19; % solids: 66.1; % N: 4.4; % S: <0.1; % Ammoniacal N: 0.17; ppm 4-Methylimidazole (4-MeI): 20; ppm 2-acetyl-4(5)-(1,2,3,4 tetrahydroxybutyl)-imidazole (THI): 23. Batch HT 2207 (CPC UK Ltd, Manchester, UK): K 610: 0.18; % solids: 70.6; % N: 4.2; % S: 0.2; % Ammoniacal N: 0.01; ppm 4-MeI: 882; ppm THI: 143. To provide a Caramel Colour III batch with a high level of

THI for research purposes, the process conditions during the preparation of batch HT 2207 were modified. Therefore, the 4-MeI and THI content of Batch HT 2207 exceed the limits defined in the ITCA specifications for Ammonia Caramel Colours.

Vitamin B6 capsules. Vitamin B6 supplementation was provided as capsules (Capsulae operculatae nr 3) containing 80 mg Cellulosum microcryst PH102 and 20 mg pyridoxine · HCl (van Lennep Apotheek, Zeist, The Netherlands), once a day from day 21 until day 33. The dose of pyridoxine corresponded with 10 times the Dutch Recommended Daily Allowance (Committee for Nutritional Norms of The Netherlands Nutrition Council, 1987) and was considered sufficient to rapidly replete vitamin B6 status.

Blood sampling. For haematology and for determination of the vitamin B6 status, 10 ml blood was collected in tubes containing ethylene-diaminetetraacetic acid-disodium salt in order to prevent coagulation. Another 10 ml blood was collected for preparation of serum for immunoglobulin-, cortisol- and acute phase protein-determinations. At the days 0, 7 and 14, an additional sample of 50 ml blood was collected in heparinised tubes for isolation of mononuclear cells for flow cytometric analysis and mitogenic stimulation assays.

Acute phase proteins C-reactive protein and α1-antitrypsin. The acute phase proteins were determined on a laser nephelometer (Behring Institute, Marburg, FRG) using rabbit antisera (Behring Institute) against C-reactive protein (CRP) and α1-antitrypsin (α1-AT).

Vitamin B6 parameters. Plasma pyridoxal 5'-phosphate (PLP) was determined according to Schrijver *et al.* (1984). The erythrocyte aspartate aminotransferase activity (EAST) and its *in vitro* stimulation by PLP (α-EAST) were determined according to Stanulovic *et al.* (1976).

Haematology. Haematological parameters were determined shortly after collection using a Sysmex K-1000 Haemoanalyser (Toa Medical Electronics Co. Ltd, Japan). The parameters recorded were: white blood cell count, lymphocyte count, neutrophil count, monocyte, basophil and eosinophil count, red blood cell count, haematocrit measurement, mean corpuscular volume, red blood cell arithmetic distribution width, haemoglobin measurement, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelet count, mean platelet volume, platelet-large cell ratio, and platelet arithmetic distribution width.

IgM, IgG and IgA determinations. Immunoglobulin levels were determined immunochemically on a laser nephelometer (Behring Institute, Marburg, BRD) using

rabbit, heavy chain specific antisera (Behring Institute). Reference-sera, calibrated against standards of the World Health Organization (WHO), were incorporated in each assay.

FACS-analysis. Mononuclear cells were enriched using Ficoll-Paque centrifugation. The isolated cells were used for lymphocyte subset-determinations by means of direct double labeling using fluorescein isothiocyanate (FITC)- and Phycoerythrin (PE)- conjugated antibodies and Flow Cytometric analysis (FACScan, Beckton Dickinson, Calif., USA). The monoclonal antibody-kits (Beckton Dickinson, Calif., USA) used were: Simultest^{TM} Control (IgG_1 FITC + IgG_{2a} PE); Simultest^{TM} LeucoGATE^{TM} (Anti-Leucocyte (CD45) FITC + Anti-Leu-M3 (CD14) PE); Simultest^{TM} T helper/suppressor test (Anti-Leu-3a (CD4) FITC + Anti-Leu-2a (CD8) PE); Simultest^{TM} Anti-Leu-4 (CD3) FITC + Anti-leu-11c (CD16) PE + Anti-Leu-19 PE; Anti-Leu-3a (CD4) PE; Anti-Leu-18 (CD45R) FITC; Anti-Leu-12 (CD19) PE and Anti-Leu-1 (CD5) FITC. The data were analysed using the Lysis-analysis system (Lysis Rev. A, 1988, Beckton Dickinson Immunocytometric Systems). Based on the side- and foreward-scatter, a gate was set for each sample, isolating the lymphocyte population. Based on the fluorescence pattern of the control sample, a cursor was set. Positive counts were those with a fluorescence level stronger than the background.

Mitogenic response assays. Isolated mononuclear cells ($5x10^5$/ml) were incubated for 96 hours at 37°C with 5% CO_2 in 200 µl RPMI medium (TNO Toxicology and Nutrition Institute, Zeist, The Netherlands) containing graded amounts of phytohaemagglutinin (PHA Reagent Grade, Wellcome Reagents Limited, Beckenham, Kent, UK; 20, 50 and 80 µg/ml), concanavalin A (Con A type IV-S, Sigma Chemical Company, St. Louis, USA; 5, 20 and 30 µg/ml) and Pokeweed mitogen (PWM, Gibco Limited, Paisley, Scotland; at dilutions of 1/6400, 1/1600 and 1/400) as well as in the absence of a mitogen. The cells were harvested after an additional incubation period of 18 hours following the administration of ^3H-Thymidine. The proliferative response was measured by determination of the amount of incorporated label using a beta-counter.

Immunoglobulin production by PWM-stimulated cells. Isolated mononuclear blood cells ($1x10^6$/ml) were incubated for 11 days at 37°C with 5% CO_2 in the presence of PWM at dilutions of 1/200 and 1/100 in RPMI medium. At the termination of the incubation, the suspensions were centrifuged at 700 g for 5 minutes and IgM, IgG and IgA levels in the supernatants were determined using an enzyme-linked immunosorbent assay (ELISA).

Cortisol determination. Cortisol levels were measured using a radio immunological method (RIA) according to the user instructions of the Coat-a-Count Cortisol RIA kit of Diagnostic Products Corporation (Los Angeles, CA, USA).

Statistical analysis. The data were analysed by analysis of variance (anova) to detect differences between the three groups. In case of significant differences according to the anova, the group means were compared by the LSD-test. Because it is known that the effects of Caramel Colour III and THI on the rat immune system are less pronounced in animals which are supplemented with vitamin B6, the results from days 3 and 7 were also evaluated by anova using the individual plasma PLP levels and the α-EAST values from day 0 as covariables to adjust for differences between the individuals at the beginning of the experimental period. The parameters studied were subject to considerable inter-individual variation. Therefore, in addition, the individual slopes of the parameter values vs. time over the experimental period (days 0, 3 and 7) were calculated and analysis was performed by anova using the individual day 0 plasma PLP levels and α-EAST values as covariables again. Data were considered significantly different when $p<0.05$.

Results

Vitamin B6 status, age distribution and body weights of the selected subjects. Based on the criteria for volunteer selection with respect to the vitamin B6 status of the participants (plasma pyridoxal 5'-phosphate [PLP] < 35 nmol/l and *in vitro* cofactor stimulation of erythrocyte aspartate aminotransferase [α-EAST] > 1.69), a test group was selected with a plasma PLP of 30 ± 9 nmol/l and an α-EAST of 1.95 ± 0.13 (mean ± SD) at the start of the study (day -14). The age of the participants was 70 ± 4, with a minimum of 65 and a maximum of 79 at the start of the study. Their body weight was 78 ± 8 kg. There were no significant differences in age distribution or in body weights among the three groups.

Acute phase protein levels, vitamin B6 supplementation and dietary reliability. In each group there was one incident of increased C-reactive protein (CRP) levels. These, however, were not correlated with changes in other parameters. Therefore, the subjects were not excluded from further calculations and statistical analyses. Abnormal α1-antitrypsin levels were not observed. Four persons did not use the vitamin B6 capsules during the end of the recovery period (from day 21 until day 33). These subjects were excluded from the calculations of the vitamin B6 parameters at

day 33. The vitamin B6 repletion clearly resulted in increased plasma PLP levels (from 25 ± 8 nmol/l at day 21 to 202 ± 83 nmol/l at day 33; $p<0.001$) and in decreased α-EAST values (from 1.93 ± 0.11 to 1.47 ± 0.09; $p<0.001$). By interviewing the participants during their regular visits to the Institute and by telephone, it appeared reasonably certain that there was good compliance with the dietary guidelines and that the desserts were consumed as planned. As a result, the test subjects are considered to have had no or only an insignificantly low uncontrolled Caramel

TABLE 7.1

TOTAL LEUKOCYTE, BLOOD NEUTROPHIL AND BLOOD LYMPHOCYTE COUNT AND B/T AND CD4+/CD8+ LYMPHOCYTE RATIOS IN CONTROL AND LOW- OR HIGH-THI CARAMEL COLOUR III-EXPOSED HUMAN VOLUNTEERS

		Cell count or ratio		Slope over exp. period adjusted for covariable	
Leukocyte subset	Group	Day 0	Day 7	PLP	α-EAST
- total leukocyte count	control	5.4 ± 1.7	5.6 ± 1.7	0.02	0.03
	low THI	5.0 ± 1.4	5.2 ± 1.5	0.04	0.03
	high THI	5.0 ± 0.7	5.1 ± 0.7	0.01	0.01
- neutrophil count	control	3.1 ± 1.3	3.3 ± 1.2	0.03	0.03
	low THI	3.2 ± 1.1	3.3 ± 1.2	0.02	0.02
	high THI	2.8 ± 0.7	2.9 ± 0.5	0.02	0.01
- lymphocyte count	control	1.7 ± 0.7	1.7 ± 0.6	- 0.01	- 0.01
	low THI	1.4 ± 0.5	1.4 ± 0.4	0.00	0.00
	high THI	1.6 ± 0.3	1.6 ± 0.4	0.00	0.00
- T/B cell ratio	control	6.07 ± 3.32	7.62 ± 3.91	0.23	0.23
	low THI	6.74 ± 2.90	9.37 ± 4.14	0.38	0.37
	high THI	5.81 ± 4.34	7.19 ± 3.81	0.19	0.19
- CD4+/CD8+ ratio	control	1.42 ± 1.34	1.53 ± 1.22	0.02	0.02
	low THI	1.51 ± 0.79	1.63 ± 0.84	0.02	0.02
	high THI	1.09 ± 0.67	0.98 ± 0.61	0.01	0.01

All data represent the mean of 8 persons per group. The data for days 0 and 7 are presented in 10^6 cells/ml blood or as ratio ± SD. The slopes are presented as the mean slope over the experimental period.

Colour III intake during the study. One person in the control group was not available at the days 21 and 33 because of family circumstances.

Blood parameters. In Tables 7.1 to 7.4, results of determinations in the test period are presented per group as mean uncorrected value ± SD at the beginning and the end of the experimental period (days 0 and 7), and as mean covariable-corrected slope over the experimental period (days 0, 3 and 7). Uncorrected values at day 3 and covariable-corrected values at days 0, 3 and 7 are not indicated in the Tables.

Results of total leukocyte, blood neutrophil and blood lymphocyte counts as well as the B/T and CD4+/CD8+ lymphocyte ratios are presented in Table 7.1. No significant Caramel Colour III intake related differences in total or differential leukocyte numbers or in B/T or CD4+/CD8+ lymphocyte ratios were detected among the groups (analysis of uncorrected as well as covariable-corrected data). Analysis of variance of the covariable-adjusted slopes over the experimental period (days 0, 3 and 7) also did not indicate an effect of exposure to Caramel Colour III on

TABLE 7.2

SERUM IMMUNOGLOBULIN LEVELS IN CONTROL AND LOW- OR HIGH-THI CARAMEL COLOUR III-EXPOSED HUMAN VOLUNTEERS

		Immunoglobulin level		Slope over exp. period adjusted for covariable	
Class	Group	Day 0	Day 7	PLP	α-EAST
IgM	control	2.3 ± 1.6	2.5 ± 1.5	0.02	0.02
	low THI	1.5 ± 1.1	1.5 ± 1.1	0.01	0.01
	high THI	1.4 ± 0.5#	1.4 ± 0.5	0.00	0.00
IgG	control	11.9 ± 3.2	12.2 ± 3.0	0.04	0.04
	low THI	12.2 ± 2.1	12.1 ± 2.2	- 0.01	- 0.01
	high THI	12.9 ± 1.1#	12.5 ± 1.6	- 0.08	- 0.07
IgA	control	3.0 ± 1.6	3.1 ± 1.7	0.02	0.02
	low THI	2.7 ± 1.1	2.7 ± 1.1	0.00	0.00
	high THI	2.6 ± 0.8#	2.4 ± 0.7	- 0.01	- 0.01

All data represent the mean of 8 or 7(#) persons per group. The data for days 0 and 7 are presented in g/l serum ± SD. The slopes are presented as the mean slope over the experimental period.

total or differential leukocyte numbers or on B/T or CD4+/CD8+ lymphocyte ratios. In addition, no effects of Caramel Colour III were detected in the analysis of results from other lymphocyte subset determinations including relative and absolute numbers of CD19+ [B] cells, CD5+/CD19- [T] cells, CD4+ T cells, CD8+ T cells, CD5- B cells, CD5+ B cells, CD4+/CD45R- [T-helper memory] cells, CD4+/CD45R+ [T-helper naive] cells, CD3+/CD16+ or CD3+/CD56+ [cytotoxic T] cells and CD3-/CD16+ or CD3-/CD56+ [Natural Killer] cells (data not presented). Results of serum immunoglobulin level determinations, presented in Table 7.2, and results from the mitogenic stimulation assays, presented as stimulation indices in Table 7.3, also indicated no significant Caramel Colour III intake related differences among the three groups.

Data on the *in vitro* immunoglobulin production by Pokeweed mitogen (PWM)-stimulated mononuclear blood cells are presented in Table 7.4. The *in vitro* IgA production in the presence of PWM at a 1/200 dilution appeared to be significantly lower in the low THI-exposed group at day 7 according to the anova & LSD-analysis. After adjustment of the data from day 7 using the day 0 α-EAST values as covariable (data not indicated in the Table), the *in vitro* IgA synthesis in the presence of PWM at a 1/200 dilution appeared to be significantly lower in both exposed groups with a mean level in the control group of 706 ng/ml supernatant, in the low THI-group of 345 ng/ml ($p<0.05$), and in the high THI-group of 419 ng/ml ($p<0.05$). After adjustment of the data from day 7 using the day 0 plasma PLP levels, no significant difference among the groups was observed in *in vitro* IgA synthesis by the mononuclear blood cells at a 1/200 dilution of PWM (data not indicated in the Table). Analyses of the data from the *in vitro* IgA synthesis in the presence of PWM at a 1/100 dilution did not indicate any differences among the three groups. Analysis of the slopes over the experimental period also did not indicate an effect of Caramel Colour III on *in vitro* IgA synthesis. No differences were detected in *in vitro* IgM or IgG production.

Results of determinations of the other haematological parameters (data on blood monocytes, basophils and eosinophils and on red blood cells, platelets and haemoglobin determinations), cortisol levels and vitamin B6 parameter did not indicate an effect of Caramel Colour III (data not presented). No significant Caramel Colour III intake related differences among the three groups were detected upon analysis of results of determinations during the recovery period (days 10, 14, 21 and 33) (data not presented).

TABLE 7.3

RESULTS OF MITOGEN INDUCED LYMPHOPROLIFERATION ASSAYS WITH BLOOD CELLS FROM CONTROL AND LOW- OR HIGH-THI CARAMEL COLOUR III-EXPOSED VOLUNTEERS

			Stimulation index (Si)		Slope over exp. period adjusted for covariable	
Mitogen	Si	Group	Day 0	Day 7	PLP	α-EAST
PHA	0 - 20 µg/ml	control	69 ± 70	44 ± 24	- 3.7	- 4.2
		low THI	74 ± 55	53 ± 47	- 3.2	- 2.6
		high THI	55 ± 49	29 ± 23	- 3.3	- 3.5
	0 - 50 µg/ml	control	203 ± 168	104 ± 52	- 14.5	- 14.8
		low THI	163 ± 98	104 ± 101	- 9.1	- 8.1
		high THI	118 ± 101	49 ± 38	- 8.9	- 9.6
	0 - 80 µg/ml	control	245 ± 183	106 ± 50	- 20.1	- 20.8
		low THI	207 ± 134	126 ± 120	- 12.0	- 10.9
		high THI	138 ± 122	62 ± 49	- 10.1	- 10.5
Con A	0 - 5 µg/ml	control	62 ± 54	79 ± 57	2.3	2.3
		low THI	67 ± 48	41 ± 27	- 4.0	- 3.5
		high THI	99 ± 61	50 ± 33	- 6.4	- 6.9
	0 - 20 µg/ml	control	89 ± 72	122 ± 83	4.4	4.6
		low THI	83 ± 58	65 ± 28	- 3.1	- 2.5
		high THI	113 ± 71	75 ± 42	- 4.6	- 5.5
	0 - 30 µg/ml	control	101 ± 51	100 ± 58	- 0.3	- 0.2
		low THI	79 ± 50	61 ± 31	- 3.0	- 2.6
		high THI	104 ± 68	68 ± 42	- 4.7	- 5.2
PWM	0 - 1/6400	control	1 ± 1	14 ± 7	1.8	1.8
		low THI	3 ± 6	11 ± 9	1.0	1.0
		high THI	1 ± 1	15 ± 12	2.0	2.0
	0 - 1/1600	control	2 ± 1	43 ± 18	5.8	5.9
		low THI	2 ± 2	28 ± 15	3.8	3.7
		high THI	3 ± 2	30 ± 26	3.9	3.9
	0 - 1/400	control	75 ± 37	48 ± 31	- 3.8	- 4.2
		low THI	53 ± 53	32 ± 19	- 2.9	- 2.8
		high THI	50 ± 29	38 ± 33	- 1.8	- 1.6

All data represent the mean of 8 persons per group. The data for days 0 and 7 are presented as Stimulation index ± SD (Si 0-X = dpm measured after incubation at concentration or dilution X of mitogen/dpm measured after incubation without mitogen). The slopes are presented as the mean slope over the experimental period.

TABLE 7.4

RESULTS OF IMMUNOGLOBULIN SYNTHESIS BY POKEWEED MITOGEN-STIMULATED BLOOD CELLS FROM CONTROL AND LOW- OR HIGH-THI CARAMEL COLOUR III-EXPOSED HUMAN VOLUNTEERS

			Immunoglobulin level		Slope over exp. period adjusted for covariable	
Class	PWM-dilution	Group	Day 0	Day 7	PLP	α-EAST
IgM	1/200	control	427 ± 428	2550 ± 1336	262.8	305.6
		low THI	727 ± 757	2002 ± 1223	89.5	63.3
		high THI	136 ± 99	2154 ± 1393	331.2	300.8
	1/100	control	110 ± 128	2589 ± 1535	392.3	460.1
		low THI	572 ± 435	1893 ± 917	167.2	161.7
		high THI	170 ± 44	1801 ± 1414	238.0	200.1
IgG	1/200	control	574 ± 408	1160 ± 636	87.2	101.9
		low THI	722 ± 445	1446 ± 389	91.9	83.6
		high THI	267 ± 202	1418 ± 508	160.7	146.7
	1/100	control	438 ± 422	1131 ± 559	108.8	127.9
		low THI	681 ± 472	1416 ± 375	104.1	106.6
		high THI	436 ± 333	1245 ± 679	112.6	86.8
IgA	1/200	control	374 ± 423	688 ± 266	49.0	46.0
		low THI	179 ± 92	350 ± 157 *	6.5	18.5
		high THI	71 ± 78	432 ± 239	77.5	68.5
	1/100	control	331 ± 402	514 ± 316	20.9	28.5
		low THI	96 ± 90	275 ± 156	14.0	23.9
		high THI	46 ± 25	265 ± 242	59.6	39.7

All data represent the mean of 8 persons per group. The data for days 0 and 7 are presented in ng/ml supernatant ± SD. The slopes are presented as the mean slope over the experimental period.

* significantly different ($p<0.05$) from control group in Anova & LSD-test.

Discussion

The results of this double-blind Caramel Colour III intervention study demonstrated that in a selected test group of apparently healthy elderly male volunteers with a biochemically marginally deficient vitamin B6 status, Caramel Colour III containing up to 143 ppm THI and administered at the level of the current Acceptable-Daily-Intake (ADI) of 200 mg/kg body weight/day during 7 days did not significantly affect any of the blood parameters determined. Although the *in vitro* IgA synthesis by mononuclear blood cells from individuals in the Caramel Colour III-exposed groups appeared to be lower in comparison to the controls in the presence of Pokeweed mitogen at a 1/200 dilution, there was no relationship between the degree of difference in IgA synthesis and the dose of THI. Moreover, comparison of the *in vitro* IgA synthesis in the control group at day 7 with the data from day 0 and day 14 (data not presented), indicated that the described differences between the groups at day 7 were likely to be due to an increased synthesis in the control group at day 7, rather than to a decrease in synthesis in the Caramel Colour III-exposed groups.

The absence of an effect in the human study was not likely to be due to low stability or to low bioavailability of THI. Because THI levels in the desserts were below the detection limit, analysis of the desserts for THI content was not possible. However, the batches of Caramel Colour III were analysed for THI several times over a longer period, and were found to be constant in THI content. Moreover, THI has been found to be a stable compound under the conditions of pH and temperature used in the study (Dr. D.V. Myers, International Technical Caramel Association, personal communications). The desserts were prepared no longer than 3 days before consumption and were stored cooled (4 to 7° C) and in dark until use. Detailed information on bioavailability of THI under varying conditions is not available. However, to the best of our knowledge, there are no reasons to expect any significant influence of the other pudding constituents in this respect. Caramel Colour III itself is a very complex mixture, containing a large number of low molecular weight compounds, as well as polymeric products (Patey *et al.*, 1985a,b; Patey *et al.*, 1987). Effects of THI administered to rats in drinking water as a Caramel Colour III solution or as an equivalent solution of synthetic THI were the same (Chapter 2). Moreover, when Caramel Colour III was administered through the diet of rats it was also found to be active (Sinkeldam *et al.*, 1988).

During the experimental period, 200 mg Caramel Colour III/kg body weight/day was consumed by all persons in the test groups, resulting in a THI intake of 28.6 μg/kg body weight/day for the high-THI Caramel Colour III group. This THI intake did not influence any of the parameters measured in these male volunteers. Data reported from rat studies show that 0.1% Caramel Colour III (THI content: 200 ppm) in drinking water is the Lowest-Observed-Effect-Level (LOEL) of THI that has been reported to induce an effect in rats (Sinkeldam *et al.*, 1988). Because effects of Caramel Colour III and THI are known to be more pronounced in rats fed diets low in vitamin B6 (Sinkeldam *et al.*, 1988; Gobin and Paine, 1989; Chapter 2), studies in which rats were fed a diet with an unspecified vitamin B6 content or deficient in vitamin B6 (< 1 ppm) were not taken into account with respect to the LOEL indicated. Based on a daily water intake of 100 g/kg body weight, the rats that received 0.1% Caramel Colour III were exposed to 100 mg Caramel Colour III/kg body weight/day, which is half of the current ADI for humans. This exposure to Caramel Colour III corresponded with an estimated daily THI intake of 20 μg/kg body weight by these rats. According to the parameters of the vitamin B6 status in the selected subjects (plasma PLP level: 30 ± 9 nMol/l and α-EAST: 1.95 ± 0.13) and the normal values of these parameters in the total Dutch population (50 ± 25 nMol/l and 1.49 ± 0.27, respectively; unpublished data, TNO-Toxicology and Nutrition Institute, Zeist, The Netherlands), the subjects that participated in the human study were considered biochemically marginally vitamin B6 deficient. The increased plasma PLP levels and decreased α-EAST values upon vitamin B6 repletion of the volunteers confirmed the relationship of these parameters with the vitamin B6 status. The animals in the study in which an estimated daily THI intake of 20 μg/kg body weight induced an effect (LOEL) were fed a diet with a vitamin B6 content of 2 to 3 ppm (Sinkeldam *et al.*, 1988). Although diets containing less than 6 ppm vitamin B6 are associated with changes in biochemical parameters in rats (National Academy of Sciences, 1978), a diet containing 2 to 3 ppm pyridoxine was not found deficient for rats on the basis of growth (Driskell *et al.*, 1973; National Academy of Sciences, 1978; Van Den Berg *et al.*, 1982) and behavioural patterns (Driskell *et al.*, 1973). Therefore, the 2-3 ppm dietary level for rats may provide a relevant basis in terms of vitamin B6 status for a comparison of data from rat studies with results in biochemically marginally vitamin B6 deficient humans. Since all effects of Caramel Colour III and THI observed in rats occur within a few days (Sinkeldam *et al.*, 1988; Chapter 5), the test period of 7 days during which Caramel Colour III was consumed in the human

study was considered sufficient to detect a potential effect. All these data concerning daily intake, vitamin B6 status and length of the experimental period together indicate that, upon oral intake and on a mg per kg body weight basis, humans are less sensitive to Caramel Colour III- or THI-induced effects than rats.

From the immunological point of view, elderly persons may not provide a representative group to study parameters of the immune system. However, because effects of Caramel Colour III and THI in rats were more pronounced in animals fed a diet relatively low in vitamin B6, a frequently occurring marginally vitamin B6 deficiency in males at an age of 65 or more (Löwik *et al.*, 1989; Bode and Van Den Berg, 1990) makes this group of elderly men a most relevant test group to study potential effects of Caramel Colour III in humans. Although the immune system of the volunteers may have been less sensitive to THI than that of rats, differences in the toxicokinetics (absorption, distribution, metabolism and/or excretion) or differences in vitamin B6 distribution and/or vitamin B6 dependence may also (in part) account for the difference in sensitivity. In particular, in dealing with extrapolation of equivalent dose levels from animal toxicity data to man, the influence of body size (scaling) has to be taken into account because extrapolation of doses on the basis of mg/kg body weight may result in an over-estimation of the effective doses in larger species (Davidson *et al.*, 1986; Vocci and Farber, 1988). With respect to toxicokinetics and possible metabolism of THI, studies in rats indicated that THI is not metabolised but is excreted rapidly in the urine (Phillips and Paine, 1990; Houben *et al.*, 1991).

Although many studies on Caramel Colour III- and THI-induced lymphopenia in rats have been conducted during the past 10 to 15 years, a consistent No-Observed-Effect-Level (NOEL) for Caramel Colour III or THI has not been reported yet. This is mainly due to the interference of vitamin B6 with the effects of Caramel Colour III and THI. Moreover, beside lymphopenia, several other effects of Caramel Colour III and THI have been detected in rats recently, including changes in immune function parameters (Chapters 2, 3 and 4). So far, there is no information on the sensitivity of the various other parameters in rat, but (immuno)histologically detectable effects on thymus and peripheral lymphoid tissues (Chapter 2) and effects observed in *ex vivo* mitogenic stimulation assays (Chapter 4) are very clear and reproducible, while several of the effects were only partially prevented by increasing the dietary vitamin B6 content to 11-12 ppm (Chapters 2 and 4). In specifying a NOEL for Caramel Colour III, not only the THI content of the Ammonia Caramel Colours but also the vitamin B6 level of the diets and/or the vitamin B6 status of the

subjects has to be considered. The Lowest-Observed-Effect-Level in rat studies reported so far suggests a No-Effect-Level for THI below 20 μg/kg body weight/day for rats fed a diet containing 2 to 3 ppm vitamin B6 (Sinkeldam *et al.*, 1988).

Caramel Colour III is commonly used in many products for human consumption (FdAC, 1987; Steering Group on Food Surveillance, 1987), and consumption of these products may result in an intake of Caramel Colour III higher than the current ADI of 200 mg/kg body weight/day. This formed the major incentive for this study in humans. The results of the presented double-blind intervention study demonstrated no effects upon oral intake of 200 mg Caramel Colour III/kg body weight/day by human subjects considered potentially sensitive to Caramel Colour III- or THI-induced changes. Since Caramel Colour III manufacturers currently meet the proposed specifications of the EC and limit THI to no more than 25 mg/kg Caramel Colour III and all effects of Caramel Colour III on the immune system can be attributed to THI (Chapter 2), the THI content of the Ammonia Caramel Colours tested (23 and 143 ppm) was sufficient to justify conclusions for Caramel Colour III in general. In addition, human exposure to THI from commercial Caramel Colour III at the current ADI of 200 mg/kg body weight/day would be less than 5 μg/kg body weight/day, whereas in the current study, the volunteers were exposed to THI-doses up to about 28 μg/kg body weight/day. Nevertheless, for the evaluation of the significance of the effects observed in rats, more information on the toxicokinetics, the mechanism of action of THI and the role of vitamin B6 will be of importance.

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Chapter 8

Summarizing Discussion

Introduction

The research described in this thesis focussed on the effects of the colour additive Caramel Colour III on the immune system. Caramel Colour III-exposure has first been associated with immunotoxicity in rats early in the 1970s. Rats exposed to this colour additive demonstrated decreased blood lymphocyte counts (Evans *et al.*, 1977; Gaunt *et al.*, 1977). Several years later, a compound with lymphopenic activity was isolated from Caramel Colour III and was identified as 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)imidazole (THI) (Kröplien *et al.* 1985). Comparative studies indicated that THI was a major, if not the only compound of Caramel Colour III, responsible for the decrease in blood lymphocyte counts (Sinkeldam *et al.*, 1988). In addition, the effects of Caramel Colour III and THI on the number of blood lymphocytes in rats were found to be more pronounced when the animals were fed a diet low in vitamin B6 (Sinkeldam *et al.*, 1988). For general information on Caramel Colours and a review of reported information about immunotoxicity of Caramel Colour III, it is referred to Chapter 1 (General Introduction) of this thesis.

The information reviewed in brief above constituted the basis of a research program aiming to characterize and compare effects of Caramel Colour III and THI on the rat immune system and to investigate the relevance of the observed changes in terms of functionality of the rat immune system. Moreover, the program aimed to generate more information with respect to the mechanism of action of Caramel Colour III and THI and the role of vitamin B6. The research was not only considered of importance for the benefit of risk assessment with respect to human intake of Caramel Colour III, but was also considered important for basic immunological and immunotoxicological knowledge. The results of the research program which are presented in this thesis are summarized and generally discussed in this Chapter.

Animal studies on Caramel Colour III and THI immunotoxicity

In Chapter 2, results of two studies conducted to characterize and compare effects of Caramel Colour III and THI on the immune system of Wistar rats and to study the influence of dietary pyridoxine (PN) on these effects are presented. No differences in effects of Caramel Colour III and THI on the rat immune system were observed. This provided further evidence for the assumption that the effects of Caramel Colour III on the immune system are caused by THI (Kröplien *et al.* 1985;

Sinkeldam *et al.*, 1988). In addition, the effects were less pronounced or absent in rats fed a diet high in PN. Whereas Caramel Colour III-exposure was associated with changes in vitamin B6 status, THI did not induce obvious effects on vitamin B6 parameters. Regarding several relationships between the effects of Caramel Colour III or THI and vitamin B6, it was proposed that the effects of Caramel Colour III and THI on the immune system result from a direct interference with a vitamin B6-dependent process, not depending on a disturbance in vitamin B6 metabolism.

Apart from reduced cell numbers in blood, spleen and lymph nodes, due to a comparable decrease in T and B lymphocyte numbers, important observations were made in the thymus of Caramel Colour III- and THI-exposed rats. In the thymus of these animals, an increase in the number of mature medullary thymocytes was observed. This observation, together with a decrease in the number of $ER4^+$ cells (recent thymus emigrants; Vaessen *et al.*, 1985) observed in the spleen of exposed rats, indicates a diminished migration of mature cells from the thymus to the periphery. A relative increase in the number of mature cells in thymus was also indicated by an increase in mitogen-induced thymidine-incorporation by thymocytes from THI-exposed rats, as described in Chapter 4. One of the most interesting observations made in Caramel Colour III- and THI-exposed rats, was a decrease in the number of macrophages positive for the antigen recognized by the monoclonal antibody ED2 in the thymic cortex and in the spleen red pulp, whereas no remarkable changes in the number of macrophages were observed at immunohistochemical examination of these tissues using other monoclonal antibodies detecting macrophages. ED2 is considered to recognize a membrane antigen (Dijkstra *et al.*, 1985) on well-differentiated macrophages, as indicated by a higher expression in a steady state population of peritoneal macrophages in comparison to inflammatory macrophages (Beelen *et al.*, 1987; Bos *et al.*, 1989). Although the expression of the antigen recognised by ED2 has not yet been clearly associated with functional characteristics of the cells, it was questioned whether a change in activity and differentiation of the total population of macrophages, as possibly indicated by a decrease in the number of $ED2^+$ macrophages, could account for the *in vivo* effects of Caramel Colour III and THI. Further evidence for an important role of $ED2^+$ macrophages in the immune system is obtained from observations in the diabetes-prone BB rat. BB rats show a strong T cell lymphopenia, comprising both the T-helper and the T-suppressor/cytotoxic subset (Elder and MacLaren, 1983; Jackson *et al.*, 1983; Yale and Marliss, 1984; Greiner *et al.*, 1986), and

interestingly, the BB rat also shows a remarkable depletion in the number of $ED2^+$ macrophages in the thymic cortex (Van Rees *et al.*, 1988).

Chapter 3 presents the results of several immune function studies with rats exposed to Caramel Colour III or THI. Evidence for a change in T-helper cell function obtained from the immune function assays is discussed in brief. In addition, an increased capacity of Caramel Colour III-exposed rats to clear *Listeria monocytogenes* bacteria shortly after infection was described. This latter effect was considered supportive for the suggested change in macrophage activity discussed in Chapter 2.

Further evidence for a change in function of accessory cells is presented in Chapter 4. In *ex vivo* studies, it was demonstrated that splenic cells from Caramel Colour III- or THI-exposed rats do not show an adequate response upon mitogenic stimulation. Although it should be recognized that due to Caramel Colour III- or THI-exposure, changes in splenic cell subpopulation distribution occur (Chapters 2, 4, and 5), the *ex vivo* effect was found to be due, at least in part, to a suppressive activity of adherent cells in the spleens from exposed animals.

Because results presented in Chapters 2, 3 and 4 indicated that the effects of Caramel Colour III and THI on the immune system may (in part) be caused by a change in activity of macrophages, future mechanistic studies on the effects of Caramel Colour III and THI on the immune system may concentrate on macrophage functions, in particular on phagocytic and killing activity, antigen processing and presentation and interleukin production. For these kind of studies, the mouse may provide a more suitable species than the rat because information on the mouse immune system is more detailed, while more experimental models and analytical techniques are available. To investigate the suitability of the mouse as a species for mechanistic studies on Caramel Colour III- or THI-induced changes in the immune system, effects of Caramel Colour III on several parameters of the immune system were studied in a pilot study with mice fed a diet containing 2 to 3 ppm vitamin B6, and the effects were compared with those observed in rats. The results presented in Chapter 6 indicate that Caramel Colour III induces similar effects in mice as it does in rats. Therefore, the mouse may be a suitable species for future mechanistic studies.

Because of the interference of vitamin B6 with the immunotoxic potency of Caramel Colour III and THI, the effects of THI on the rat immune system have been compared with the effects of the vitamin B6 antagonist 4'-deoxypyridoxine (DOP). The results presented in Chapter 5 demonstrated that effects of THI on the rat immune system are similar to those of DOP. Moreover, time-sequence studies on the

effects of THI and DOP presented in Chapter 5 also demonstrated a comparable time-dependence of the THI- and DOP-induced changes in the rat immune system. Based on the similarity in effects of THI and DOP on the rat immune system and the protective potency of vitamin B6, it was suggested that THI and DOP share a same mechanism of immunotoxicity, accomplished by inhibition of vitamin B6-dependent processes. Because THI does not affect vitamin B6 metabolism and it was indicated that effects of DOP on the immune system do not necessarily depend on a decrease in pyridoxal 5'-phosphate (PLP) levels, it was hypothesized that the THI- and DOP-induced inhibition of vitamin B6-dependent processes is mainly due to a competition between THI or DOP 5'-phosphate and the coenzyme PLP for binding to the cofactor binding-site of one or more PLP-dependent enzymes (see also the paragraph: mechanistic considerations and future research). Since in this hypothesis, an anti-vitamin B6 action is proposed to be the primary mode of action of Caramel Colour III and THI, special attention has to be paid to what is known about effects of vitamin B6 deficiency, particularly with respect to roles of vitamin B6 and vitamin B6-dependent processes in immune functions. For more general information on vitamin B6, it is referred to the General Introduction in Chapter 1 of this thesis.

Animal studies on roles of vitamin B6 in immune functions

Information on the importance of vitamin B6 in immune functions is more detailed from animal studies than from human studies or clinical conditions. Unfortunately, in most of the animal studies reported, deficiency was promoted by the addition of DOP or other vitamin B6 antagonists to the diet or drinking water of the animals, which makes the status of the animals not necessarily comparable to a real deficiency state.

Robson and Schwarz (1975a) reported decreased blood lymphocyte counts, increased numbers of blood neutrophils, and decreased numbers of cells in thoracic duct lymph in DOP-exposed rats fed a diet deficient in vitamin B6. The capacity of the thoracic duct lymph cells to respond in the mixed lymphocyte reaction (MLR), to produce a lymphocyte transfer reaction, and to incorporate labeled uridine *in vitro* was impaired (Robson and Schwarz, 1975a). Chandra and Puri (1985) described a decreased lymphocyte response to mitogenic stimulation with phytohaemagglutinin for DOP-exposed rats fed a diet deficient in vitamin B6. Sergeev *et al.* (1978) demonstrated decreased spleen weights and impaired MLR and cytotoxicity of

spleen lymphocytes from mice fed a diet deficient in vitamin B6 and exposed to isonicotinylhydrazide, a carboxyl-trapping vitamin B6 antagonist. Blood and spleen cell lymphoproliferation in response to stimulation with phytohaemagglutinin or concanavalin A was higher in mice fed diets with increasing levels of vitamin B6 (Gridley *et al.*, 1987), and *in vivo* and *in vitro* stimulation of cell mediated immunity by vitamin B6 was further demonstrated for mice by Gridley *et al.* (1988). Thymus weights are markedly decreased in DOP-exposed rats fed a vitamin B6 deficient diet (Chandra and Puri, 1985). A possible role of vitamin B6 in thymic epithelial cell function and T cell maturation has been indicated. Exposure of lymphoid precursors from neonatally thymectomized or vitamin B6 deficient DOP-exposed donor rats to normal thymic epithelial cell monolayers resulted in their conversion to functional T lymphocytes as measured by their response in the MLR and to mitogens, whereas thymic epithelial cell monolayers from vitamin B6 deficient DOP-exposed rats were not able to effect such a maturation of T lymphocytes (Willis-Carr and Pierre, 1978). These data were confirmed by Chandra and Puri, who also demonstrated decreased thymic hormone activity in serum of vitamin B6 deficient DOP-exposed rats (Chandra and Puri, 1985). In addition, the response of spleen cells from vitamin B6 deficient DOP-exposed rats to stimulation with phytohaemagglutinin or concanavalin A was impaired (Chandra and Puri, 1985). The response of these cells to mitogenic stimulation was restored by co-culturing with normal thymic epithelium, whereas thymic epithelium from vitamin B6 deficient DOP-exposed rats was inactive (Chandra and Puri, 1985). Phagocytosis of opsonized sheep red blood cells by alveolar macrophages of rats fed a vitamin B6 deficient diet for 4 weeks was slightly increased (Moriguchi and Kishino, 1984). However, enhancement of phagocytic activity of the alveolar macrophages from the vitamin B6 deficient rats by incubation with a macrophage-activating factor was markedly impaired (Moriguchi and Kishino, 1984). In addition, macrophage-activating factor production by splenic cell suspensions from the deficient rats was impaired (Moriguchi and Kishino, 1984). Although the number of cells recovered from the peritoneal cavity was not affected in mice due to vitamin B6 deficiency, macrophages were found to comprise a higher percentage of the peritoneal exudate cell population in vitamin B6 deficient mice (Ha *et al.*, 1984). This resulted in a slightly increased phagocytosis of nonopsonized and opsonized sheep red blood cells by peritoneal macrophages from these vitamin B6 deficient mice (Ha *et al.*, 1984). Survival times of skin homografts (Axelrod *et al.*, 1958) and renal transplants (Dobbelstein *et al.*, 1979) were increased markedly in rats

fed a B6 deficient diet and exposed to DOP. High dietary intake of vitamin B6 suppressed tumor development in mice possibly by enhanced immune status, although PLP growth regulation of the tumor may have played a role (Gridley *et al.*, 1987). Primary as well as secondary splenic and peritoneal T-cell-mediated cytotoxicity against P815 tumor cells was depressed in vitamin B6 deficient mice (Ha *et al.*, 1984). Complement-dependent antibody-mediated cytotoxicity against P815 cells, and naive and interferon-induced Natural Killer cell activity against YAC cells were not affected in the deficient mice (Ha *et al.*, 1984). Reduced numbers of antibody-forming cells and/or decreased antibody levels in blood were reported in vitamin B6 deficient rats (Gershoff *et al.*, 1968; Debes and Kirksey, 1979) and in vitamin B6 deficient rats exposed to DOP (Gershoff *et al.*, 1968; Chandra and Puri, 1985).

Effects of vitamin B6 deficiency either or not in combination with DOP-exposure during pregnancy and lactation on immune capacities of the offspring have been reported by several groups. Rat studies indicated that passive antibody transfer from dams to pups was not influenced by vitamin B6 deficiency (Debes and Kirksey, 1979). However, immune capacities of the offspring may be affected by congenital vitamin B6 deficiency, as reported by Davis (1974). Pregnant rats were fed a diet deficient in vitamin B6 and were exposed to DOP in drinking water from day 4, 5 or 6 to day 21 of gestation, when they were returned to regular diet and tap water. The newborn rats were weaned at 28 days of age and received regular diet and tap water. At 6 weeks of age, several immune function assays in the offspring were started. Delayed hypersensitivity reactions in the offspring were markedly suppressed. Primary and secondary antibody responses to sheep red blood cell and *Escherichia coli* antigens in the offspring were not depressed. Robson and Schwartz (1975b) demonstrated that the immunodeficiency from such a congenital vitamin B6 deficiency persisted for at least 3 months. Lymphocyte and neutrophil numbers in peripheral blood of offspring from control mothers or mothers fed a vitamin B6 deficient diet and exposed to DOP from day 4, 5 or 6 until day 21 of pregnancy did not differ 3 months after birth. While the number of cells in thoracic duct lymph of the offspring of the vitamin B6 deficient DOP-exposed mothers was also nearly equivalent to control values at 3 months of age, such cells had a reduced capacity to respond in the MLR and in lymphocyte transfer reaction.

Vitamin B6 status in humans; Relationships with clinical conditions and immune functions

Vitamin B6 plays a role in many metabolic processes, including many of the enzyme-catalysed reactions of amino acids like decarboxylations, transaminations, racemizations, β- and γ-eliminations and several others, thereby indirectly influencing many other processes (see for a recent review: Friedrich, 1988). In addition, many not directly PLP-dependent enzymes or processes were found to be influenced by PLP (Friedrich, 1988). Because vitamin B6 plays such a central role in metabolism, food products from plant and animal origin contain quite some vitamin B6. Whereas pyridoxine (PN) is the most common form of vitamin B6 in plant products, in products from animal origin the vitamin occurs primarily as pyridoxal (PL) and pyridoxamine (PM) (Friedrich, 1988). As indicated already in the General Introduction (Chapter 1), phosphate ester forms of vitamin B6 present in food are hydrolysed in the lumen of the intestinal tract and the non-phosphorylated derivatives are rapidly absorbed. The availability of vitamin B6 from food may be decreased by storage and heat (Friedrich, 1988). In food products that have been exposed to high temperatures during processing, vitamin B6 may partially be bound to cysteine and lysine residues of amino acids and proteins. Although the bioavailability of vitamin B6 from such condensation products is impaired, partial release of bound vitamin B6, possibly by *in vivo* enzymatic hydrolysis, was demonstrated for ε-pyridoxyllysine, resulting in 60% biological activity relative to the molar potency of pyridoxine (Gregory, 1978). Many natural antagonists of vitamin B6 are known, the majority of them are carboxyl reagents like hydrazines or hydroxylamines, being active due to the formation of complexes with pyridoxal (PL) or PLP (Sauberlich, 1968; Kierska and Maslinski, 1971; Klosterman, 1974; Kierska *et al.*, 1978; Klosterman, 1979; Klosterman, 1981). Several structural analogs of vitamin B6 exhibit antagonistic activity because they do not contain the structural features for catalytic action, but inhibit vitamin B6 metabolizing or vitamin B6-dependent enzymes (Sauberlich, 1968; Friedrich, 1988). However, presumably partly due to the general presence of vitamin B6 in biological products, spontaneous deficiency in humans is not seen frequently (World Health Organization, 1974). Nevertheless, (marginally) vitamin B6 deficiency may occur under unusual conditions, for instance, secondary to disease or inborn errors in metabolism (World Health Organization, 1974). Several aspects of human requirements for vitamin B6 are reviewed in reports of the National Academy of Sciences (1978 and 1980).

Several human populations at increased risk for a (marginally) deficiency in vitamin B6 have been indicated. Among these are, for instance, persons at chronic alcohol abuse, females on oral contraceptives, pregnant women, breast-fed infants, and elderly women and men (Klosterman, 1981; Wilson and Davis, 1983; Driskell, 1984; Ink and Henderson, 1984; Friedrich, 1988). Moreover, several clinical conditions in humans were reported to be improved as a response to (high doses of) vitamin B6, including some neonatal convulsions and genetic conditions like vitamin B6-responsive anemia and hyperoxaluria (Wilson and Davis, 1983; Ink and Henderson, 1984; Friedrich, 1988). In addition, there have been some reports on relationships between the vitamin B6 status and immune functions in humans. For instance, vitamin B6 supplementation reversed diminished lymphocyte reactivity in uremia patients (Dobbelstein *et al.*, 1974), improved immune functions in patients undergoing hemodialysis (Casciato *et al.*, 1984; Kamata *et al.*, 1979), and resulted in increased lymphocyte proliferation in response to mitogenic stimulation in a group of persons aged 61-81 years (Talbott *et al.*, 1987). In these elderly persons, percentages of CD3+ and CD4+ cells in blood were increased, whereas the percentage of CD8+ cells was not affected upon pyridoxine supplementation (Talbott *et al.*, 1987). In addition, reduced numbers of circulating lymphocytes were observed in humans fed a diet low in vitamin B6 (Cheslock and McCully, 1960). However, care must be taken in administering (high doses of) vitamin B6 because of possible adverse effects, in particular neurotoxicity (Phillips *et al.*, 1978; Schaumburg *et al.*, 1983; Driskell, 1984; Parry and Bredesen, 1985. Friedrich, 1988; Krinke and Fitzgerald, 1988). In contrast to improved immune functions in response to vitamin B6 supplementation, short term vitamin B6 restriction, which induced a state of marginal vitamin B6 deficiency, did not affect immune functions (Van Den Berg *et al.*,1988). In addition, no differences in immune function parameters were observed between healthy elderly persons receiving high and low vitamin B6 intakes (Goodwin and Garry, 1983). Exposure of humans to DOP has also been reported to induce lymphopenia (Vilter *et al.*,1953; Wayne *et al.*, 1985). Antibody production was only mildly impaired in DOP-exposed humans (Hodges *et al.*, 1962).

Roles of vitamin B6 in immune functions and effects of vitamin B6 deficiency, DOP, Caramel Colour III and THI on the immune system; Mechanistic considerations and future research

Although from the previous paragraphs it is obvious that vitamin B6 plays an important role in immune functions, the critical processes in which it is involved in immune functions are as yet unknown. As already indicated, vitamin B6 plays a role in many metabolic processes. Because vitamin B6 plays such a central role in metabolism, interference by deficiency or antagonists of the vitamin with immune functions may take place at many levels. Special attention has been paid to the role of PLP in the production of "active formaldehyde" via the PLP-dependent glycine-serine interconversion, thereby affecting nucleic acid metabolism (Trakatellis and Axelrod, 1965). Interestingly, decreased antibody production in DOP-exposed rats fed a diet deficient in vitamin B6 was partially reversed by increasing the dietary glycine or serine contents (Gershoff *et al.*, 1968). Several studies have been conducted on the interference of vitamin B6 with steroid hormone action. PLP was demonstrated to inhibit steroid-receptor complex binding to DNA, probably through Schiff's base formation with the activated cytosolic complex, and as a consequence, vitamin B6 deficiency enhances end-organ and cell sensitivity to steroid hormones *in vivo* and *in vitro* (Bowden *et al.*, 1986; Compton and Cidlowski, 1986; Bender *et al.*, 1988).

The major mechanism(s) primarily involved in immunomodulation by DOP are also unknown. As described in Chapter 5, DOP competes with PLP-precursors for phosphorylation by pyridoxal kinase and, as a consequence, DOP may induce a decrease in PLP levels. However, effects of DOP on rat lymphocyte counts were reported at doses that did not affect plasma PLP levels (Sinkeldam *et al.*, 1988), while in rats fed a diet low in vitamin B6, exposure to DOP does not necessarily induce a decrease in tissue PLP levels but may even increase tissue PLP contents (Johnston *et al.*, 1966; Coburn *et al.*, 1981). Evidence for a significant role of competition between DOP 5'-phosphate and the coenzyme PLP for binding to the cofactor binding-site of PLP-dependent enzymes was discussed in Chapter 5. Based on the information available, it was proposed in that Chapter that the effects of DOP on the immune system are mainly due to the direct inhibition of PLP-dependent enzymes caused by this competition between DOP 5'-phosphate and PLP, as was previously indicated in this Summarizing Discussion. Further, it was proposed in Chapter 5 that the same mechanism of action underlies the effects of THI on the immune system.

Unfortunately, there is not much information about roles of vitamin B6-dependent enzymes in immune functions, but the importance of PLP in the activity of ornithine decarboxylase and decarboxylases involved in the production of mediators such as histamine and serotonin was indicated already in Chapter 5. For ornithine decarboxylase it is known that it is involved in many immunological processes, including lymphocyte and macrophage activation, as well as production of, and responsiveness to cytokines (Fidelus *et al.*, 1984; Kierszenbaum *et al.*, 1987; Endo *et al.*, 1988; Farrar *et al.*, 1988; Schall *et al.*, 1991).

Currently, studies are in progress to investigate effects of DOP, DOP 5'-phosphate and THI on several PLP-dependent enzymatic reactions using *in vitro* assays with isolated enzyme systems. However, in Chapter 5 it was also indicated that although potentially, all enzymatic reactions in which PLP acts as a coenzyme may be suppressed due to the proposed action of DOP 5'-phosphate and possibly THI, the effects of THI and DOP on the immune system may likely result from interference with one or several sensitive enzymes or processes. Differences in affinity of binding to the cofactor binding site of the apo-enzymes (Korytnyk *et al.*, 1976; Snell, 1970) may contribute to this selectivity. Furthermore, differences in affinity may also result in a different spectrum of inhibition of vitamin B6 dependent enzymes by THI, in comparison to vitamin B6 deficiency or DOP-exposure. The latter may explain why classical signs of a vitamin B6 deficiency, which may also be induced by DOP, were never observed in toxicity studies with Caramel Colour III and THI. Future studies on the significance of an anti-vitamin B6 action of DOP and THI in their immunomodulation may also focus on a comparison with effects of other vitamin B6 antagonists on the immune system. In particular, data on effects of carboxyl-trapping antagonists like hydrazines or hydroxylamines may be of interest. Such compounds that are present in many natural products (Sauberlich, 1968; Kierska and Maslinski, 1971; Klosterman, 1974; Kierska *et al.*, 1978; Klosterman, 1979; Klosterman, 1981), exhibit vitamin B6 antagonistic activity due to an other mechanism than antagonistic vitamin B6 analogs like DOP, because they do not really compete with PLP for binding to enzymes, but they bind to PL or PLP, thereby inactivating PLP and PLP-dependent enzymes. The effects of DOP and THI described in this thesis and results from studies with other vitamin B6 antagonists may provide new information on roles of vitamin B6 in immune functions.

In addition to biochemical interferences possibly involved in the immunomodulation by DOP and THI discussed in Chapter 5 and in the previous

sections of this Summarizing Discussion, it was suggested in Chapters 2, 3 and 4 that the effects of Caramel Colour III and THI on the immune system may be caused by a change in activity of macrophages. Therefore, future mechanistic studies on the effects of Caramel Colour III and THI on the immune system may concentrate on macrophage functions, in particular on phagocytic and killing activity, antigen processing and presentation and interleukin production.

No-Observed-Effect-Levels in rat studies and experimental and dietary Caramel Colour III-exposure of humans

Although many studies on Caramel Colour III- and THI-induced lymphopenia in rats have been conducted during the past 10 to 15 years, a consistent No-Observed-Effect-Level (NOEL) for Caramel Colour III or THI has not been reported yet. This is mainly due to the interference of vitamin B6 with the effects of Caramel Colour III and THI. Moreover, the NOEL may depend on the parameters investigated. Beside lymphopenia, several other effects of Caramel Colour III and THI have been detected in rats recently, including changes in immune function parameters (see Chapters 2, 3, 4 and 5). So far, there is no information on the sensitivity of the various other parameters in rat, but the (immuno)histologically detectable effects on thymus and peripheral lymphoid tissues (Chapter 2) and the effects observed in *ex vivo* mitogenic stimulation assays (Chapter 4) are very clear and reproducible, while several of the effects of Caramel Colour III and THI were only partially prevented by increasing the dietary vitamin B6 content to 11-12 ppm (Chapters 2 and 4). In specifying a NOEL for Caramel Colour III, not only the THI content of the Ammonia Caramel Colours but also the vitamin B6 level of the diets and/or the vitamin B6 status of the subjects has to be considered. The Lowest-Observed-Effect-Level in rat studies reported so far suggests a No-Effect-Level for THI below 20 μg/kg body weight/day for rats fed a diet containing 2 to 3 ppm vitamin B6 (Sinkeldam *et al.*, 1988). For a Caramel Colour III containing 25 ppm THI (see remark below), this would be equivalent to a Caramel Colour III intake below 800 mg/kg body weight/day.

Caramel Colour III is commonly used in many products for human consumption, including various bakery products, soya-bean sauces, brown sauces, gravies, soup aromas, brown (dehydrated) soups, brown malt caramel blend for various applications, vinegars and beers, particularly in certain dark-brown beers

(FdAC, 1987; Steering Group on Food Surveillance, 1987). Consumption of these products may result in an intake of Caramel Colour III higher than the current Acceptable Daily Intake (ADI) of 200 mg/kg body weight/day. Effects in (marginally vitamin B6 deficient) humans upon dietary intake of Caramel Colour III can not be excluded. This formed the major incentive to conduct a human study. The results of the double-blind intervention study presented in Chapter 7 demonstrated no effects upon oral intake of 200 mg Caramel Colour III/kg body weight/day (equivalent to 28 μg THI/kg body weight/day for the Caramel Colour III batch containing 143 ppm THI) by human subjects considered potentially sensitive to Caramel Colour III- or THI-induced changes because of a biochemically marginally vitamin B6 deficiency.

With respect to dietary intake of Caramel Colour III by humans it must be recognized that Caramel Colour III manufacturers currently meet the proposed specifications of the EC and limit THI to no more than 25 mg/kg Caramel Colour III. Therefore, and because all effects of Caramel Colour III on the immune system can be attributed to THI (Chapter 2), the THI content of the Ammonia Caramel Colours tested (23 and 143 ppm) was sufficient to justify conclusions for Caramel Colour III in general. In addition, human exposure to THI from such a Caramel Colour III (25 ppm THI or below) at the current ADI would be 5 μg/kg body weight/day or below, while in the intervention study, the volunteers were exposed to levels up to 28 μg/kg body weight/day. Nevertheless, for the evaluation of the significance of the effects observed in rats, more information on the toxicokinetics, the mechanism of action of THI and the role of vitamin B6 will be of importance.

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Korte samenvatting

Vitamine B6 Status-Afhankelijke Immunomodulatie door Caramel Kleurstof III

Inleiding

Met de term caramels (E150) wordt een grote groep producten aangeduid die als kleurstof worden toegevoegd aan verschillende voedingsmiddelen en dranken voor humaan gebruik. Caramels vertegenwoordigen op gewichtsbasis naar schatting ruim 95% van de totale hoeveelheid voor humane voedingsmiddelen gebruikte kleurstoffen. Caramels worden bereid via gecontroleerde verhitting van koolhydraten onder aanwezigheid van verschillende reactanten. Afhankelijk van de tijdens de productie aanwezige reactanten worden caramels ingedeeld in 4 klassen. Het in dit proefschrift beschreven onderzoek was gericht op effecten van caramels behorende tot de derde klasse: de Ammonia Caramels (Caramel Kleurstof III).

Caramel Kleurstof III wordt onder andere verwerkt in diverse bakkerijproducten, sauzen, soepen en soeparoma's, azijn en bieren, in het bijzonder donkere bieren. In Europa behoort naar schatting ongeveer 50% van de gebruikte caramels tot de Ammonia Caramels, terwijl in de Verenigde Staten het percentage tussen de 20 en 25 ligt. Begin zeventiger jaren werd ontdekt dat Caramel Kleurstof III toegediend aan ratten via voeding of drinkwater een daling in het aantal witte bloedcellen kan veroorzaken. Deze daling bleek toe te schrijven aan een afname in het aantal lymfocyten. Verder onderzoek wees uit dat de mate van lymfopenie in sterke mate afhankelijk is van de vitamine B6 status van de proefdieren. Caramel Kleurstof III belasting gecombineerd met een laag, doch niet deficiënt vitamine B6 gehalte van het dieet, leidt tot een sterke lymfopenie. Bij een hoger gehalte aan vitamine B6 is de daling in het aantal perifere bloedlymfocyten minder sterk. Tevens werd vastgesteld dat waarschijnlijk één verbinding in Caramel Kleurstof III verantwoordelijk is voor de immunomodulerende werking: het 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazol (THI).

De in voorgaande alinea beschreven informatie vormde de aanleiding tot de start van het onderzoeksproject waarvan de resultaten in dit proefschrift zijn weergegeven. Het onderzoek had onder andere tot doel effecten van Caramel Kleurstof III en THI op het immuunsysteem van ratten zo nauwkeurig mogelijk te beschrijven en te vergelijken. Daarnaast was het onderzoek erop gericht om door middel van immuunfunctie-onderzoek functionele consequenties van de waargenomen effecten te analyseren en het mechanisme via welke de effecten tot stand komen en de rol van vitamine B6 hierin zo mogelijk te verklaren. Een beter inzicht in de immunotoxiciteit van Caramel Kleurstof III werd niet alleen van belang geacht voor de evaluatie van eventuele risico's van blootstelling van mensen aan dit voedseladditief, maar werd tevens van belang geacht voor algemene immunologische en immunotoxicologische kennis.

De inhoud van dit proefschrift

Het onderzoek beschreven in dit proefschrift toonde aan dat Caramel Kleurstof III en THI identieke veranderingen in het immuunsysteem van ratten veroorzaken, hetgeen aangeeft dat de effecten van het kleuradditief inderdaad door THI veroorzaakt worden. Naast effecten op het aantal bloedlymfocyten bleken onder invloed van Caramel Kleurstof III en THI ook veranderingen op te treden in diverse andere organen die deel uitmaken van het immuunsysteem, zoals de milt en lymfklieren. Deze veranderingen werden eveneens voornamelijk gekenmerkt door een afname in het aantal lymfocyten. Daarnaast werden veranderingen aangetoond in de thymus (zwezerik) van Caramel Kleurstof III- en THI-behandelde dieren en werden veranderingen in macrofaag-populaties waargenomen. Van dit laatste celtype, de macrofaag, is bekend dat het een belangrijke rol speelt bij de regulatie van activiteiten van het immuunsysteem. Alhoewel de waargenomen effecten niet, of slechts zeer zwak bleken op te treden in proefdieren die een dieet ontvingen met een hoog gehalte aan vitamine B6, leverde een goede vitamine B6 status geen volledige garantie voor het uitblijven van effecten.

Immuunfuctie-studies met ratten toonden aan dat onder invloed van Caramel Kleurstof III en THI verschillende functies van het immuunsysteem afgenomen waren. Een andere functie echter, de afweer tegen de intracellulaire bacterie de *Listeria monocytogenes*, bleek toegenomen onder invloed van Caramel Kleurstof III. De toegenomen activiteit van macrofagen tegen de bacterie *Listeria monocytogenes*

houdt mogelijk verband met de waargenomen veranderingen in macrofaag-populaties. Aanwijzingen voor veranderingen in activiteit van adherente cellen werden verder verkregen uit studies waarbij gebruik gemaakt werd van miltcellen van Caramel Kleurstof III- en THI-behandelde ratten. Miltcellen van belaste dieren bleken namelijk niet of slechts zeer zwak te reageren op een mitogene stimulatie tot deling. Deze verstoring bleek (deels ?) veroorzaakt te worden door een remmende werking van adherente cellen in de milt van deze behandelde dieren.

Caramel Kleurstof III wordt veelvuldig toegepast in voedingsmiddelen en alcoholische dranken. In sommige biersoorten komen ze zelfs in zeer hoge concentraties (tot 12 á 15 %) voor. Consumptie van deze bieren levert al snel een opname van Caramel Kleurstof III boven de huidige aanvaardbaar geachte dagelijkse opname, de "Acceptable-Daily-Intake" (ADI), van 200 mg per kg lichaamsgewicht per dag. De bijdrage van andere consumptie-artikelen aan de opname van Caramel Kleurstof III kan eveneens aanzienlijk zijn. In verband met de mogelijk hoge opname van Caramel Kleurstof III via consumptie-artikelen zijn effecten in de mens niet uit te sluiten. Dit vormde de belangrijkste aanleiding voor het uitvoeren van een interventiestudie naar eventuele effecten van Caramel Kleurstof III in de mens. In de interventiestudie die in dit proefschrift is beschreven werden geen effecten waargenomen ten gevolge van Caramel Kleurstof III-belasting op een niveau van de huidige ADI van 200 mg per kg lichaamsgewicht per dag in een groep vrijwilligers behorende tot een potentiële risicogroep voor een effect van Caramel Kleurstof III. Desalniettemin wordt aangegeven dat nader onderzoek van belang is om eventuele risico's van blootstelling van mensen aan Caramel Kleurstof III via consumptie-artikelen optimaal in te kunnen schatten.

Met betrekking tot opheldering van het mechanisme via welke de effecten tot stand komen en de rol van vitamine B6 hierin wordt eveneens aangegeven dat nader onderzoek nodig zal zijn. In het proefschrift wordt beschreven dat veranderingen in macrofagen mogelijk een belangrijre rol spelen bij het tot stand komen van de diverse effecten die zijn waargenomen. Daarnaast wordt aangegeven dat de effecten wellicht veroorzaakt worden door een verstoring van één of meer vitamine B6-afhankelijke processen. Het feit dat een bekende vitamine B6 antagonist, het 4'-deoxypyridoxine (DOP), overeenkomstige veranderingen in het immuunsysteem van ratten teweeg blijkt te brengen wordt als een nieuwe ingang voor toekomstig onderzoek aangegrepen.

Curriculum vitae

Geert Houben werd op 12 december 1962 te Sittard geboren. Na de lagere school heeft hij tussen 1975 en 1981 Voorbereidend Wetenschappelijk Onderwijs gevolgd aan de SERVIAM R.K. Scholengemeenschap Lyceum-HAVO te Sittard. In 1981 behaalde hij het Atheneum B diploma (met lof), waarna in datzelfde jaar begonnen werd met de studie Biologie aan de Rijksuniversiteit Utrecht (RUU). Tijdens de kandidaatsfase werd de specialisatie-richting Medische Biologie (B5*) gekozen en in 1985 werd het kandidaatsexamen afgelegd. Tijdens de doctoraalfase werd een bijvak Toxicologie (Prof. dr. R.A.A. Maes, faculteit Farmacie, Rijksuniversiteit Utrecht), een hoofdvak Immunologie, richting Allergologie (Prof. dr. L. Berrens, faculteit Geneeskunde, Rijksuniversiteit Utrecht) en een hoofdvak Biologische Toxicologie, richting Immunotoxicologie (Prof. dr. W. Seinen, faculteiten Biologie en Diergeneeskunde, Rijksuniversiteit Utrecht) gevolgd. Het doctoraalexamen werd in 1987 cum laude afgelegd.

Vanaf october 1987 tot en met september 1991 was hij als promovendus werkzaam binnen het Utrechts Toxicologisch Centrum (UTOX), een samenwerkingsverband van de Rijksuniversiteit Utrecht, de Nederlandse Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek (TNO) en het Rijksinstituut voor Volksgezondheid en Milieuhygiëne (RIVM). Tevens werd in deze periode de Postdoctorale Opleiding Toxicologie gevolgd.

Vanaf september 1991 was hij als Toegevoegd Onderzoeker werkzaam binnen het Research Instituut Toxicologie (RITOX) van de Rijksuniversiteit Utrecht en sinds maart 1992 is hij als Toegevoegd Onderzoeker tewerkgesteld bij het TNO Instituut voor Toxicologie en Voeding (TNO ITV) in Zeist.

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